

# A study of low voltage polyacrylamide gel electrophoresis as a means of providing controlled drug release

Kumar, Ravinder

*Award date:*
1986

*Awarding institution:*
University of Bath

# A Study of Low Voltage Polyacrylamide Gel Electrophoresis as a means of providing Controlled Drug Release

A thesis submitted by

**Ravinder Kumar**

for the degree of Doctor of Philosophy

of the University of Bath

1986







ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

**Acknowledgements**

The author would like to thank his academic supervisor, Dr J N Staniforth, for his friendship, guidance and encouragement throughout this study. The support of Professor J E Rees is also appreciated.

The helpful discussions with the author's industrial supervisor, Mr R Marsden, and also Dr J Lechleiter are deeply appreciated.

The author would like to thank Mr G Venn, Bath University Science Area Workshop for advice relating to construction of some of the apparatus used in his study.

The Staff at the Pathology department, Royal United Hospital, Bath, are thanked for their co-operation in supplying skin samples used in this work.

The assistance of Mrs K Powell of the Electron Optics Centre, University of Bath, is also acknowledged.

Dawn Sexton is thanked for the typing of this manuscript.

Lastly, the author would like to thank the University of Bath and Eli Lilly and Company for funding this work.

To my parents and most of all, to my wife, for suffering so many lonely evenings and weekends.

## SUMMARY

A study of low voltage polyacrylamide gel electrophoresis (PAGE) as a basis for providing controlled drug release has been carried out.

Polyacrylamide gels were prepared by chemically initiated polymerization and were found to swell and leach U.V. absorbing materials in aqueous solutions. The degree of swelling was influenced by polymerization temperature, gel formulation and ionic strength of immersing solution. Swelling of polyacrylamide gel was found to be negligible during electrophoresis.

Rod PAGE showed that it was possible to use lower voltages (e.g. 5V) than conventional PAGE techniques (typically 100V) to effect reproducible electrophoretic migration of proteins such as bovine serum albumin and insulin; protein migration was influenced by gel formulation and electrolyte buffer ionic strength and pH.

It was found that permeation of drugs such as insulin, hyoscine HBr, propranolol HCl and hyoscine methyl chloride through polyacrylamide, could be reduced by manipulation of gel formulation. Thus polyacrylamide gel discs were used to provide a "barrier" to drug permeation from reservoir solutions. Subsequent application of an electric field allowed drug to be eluted from the reservoir.

By use of a stabilized power supply providing constant voltage conditions (e.g. 5V), the above drugs could be released at constant rates for several days. In addition, insulin and hyoscine methyl chloride could be released intermittently by removal and re-application of the electric field, with no lag time observed. Drug release rates were influenced by applied voltage, temperature and polyacrylamide gel formulation.

A study of *in vitro* permeation of hyoscine hydrobromide through human skin indicated that permeation rate was influenced by donor drug solution concentration and pH. Some factors important in a 3-compartment model intended for study of controlled *in vitro* electrophoretic drug delivery to human skin were investigated.

It was concluded that low voltage PAGE appears to be a promising means of providing controlled drug release.

CONTENTS

# 1. INTRODUCTION

## 1.1. Controlled drug delivery

Most drugs are currently administered either parenterally or by mouth; a typical plasma concentration versus time profile following a single oral bolus of drug is shown in figure 1.1a. Plasma drug concentration rises initially, peaks, and gradually falls as drug is metabolised and excreted. Depending on a drug's biological half life and its absorption, distribution and elimination characteristics [1], repetitive dosing in the above manner may result in a "pulsing" plasma concentration profile (figure 1.1b) where plasma drug levels alternate between those causing toxicity and those having no therapeutic action.

"Slow" or "sustained" release preparations have been formulated to achieve more desirable patterns of drug release e.g. by use of enteric coating agents and special tableting excipients [2]. However most of these methods, which retard or slow drug release are influenced by environmental conditions and lack inter-patient reproducibility [3,27]. Most workers agree that delivery of drugs at constant (or zero-order) rates is desirable for the purpose of maintaining plasma levels within the "therapeutic window" [4,5,6]. Not all workers agree that zero-order drug delivery is optimal, for example Wood [7] suggests that first-order release kinetics as observed in matrix delivery devices (section 1.4.1.2.) are desirable in some circumstances.

Devices capable of releasing drug in a "controlled" manner for prolonged time periods have been termed "controlled release" systems. Certain disease states require more complex dosage regimens, e.g. diabetes mellitus where a basal insulin level needs to be supplemented post-prandially. Sustained and controlled drug release has been the subject of numerous publications [3,5,8-18].

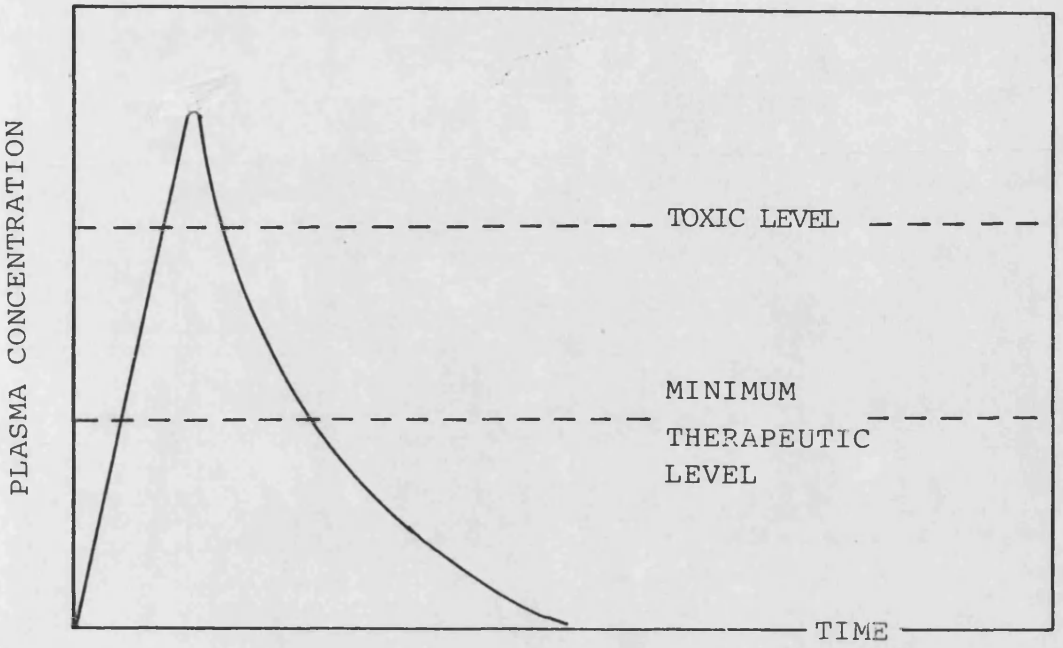



FIGURE 1.1a TYPICAL PLASMA CONCENTRATION VERSUS TIME PROFILE FOLLOWING SINGLE ORAL BOLUS OF DRUG

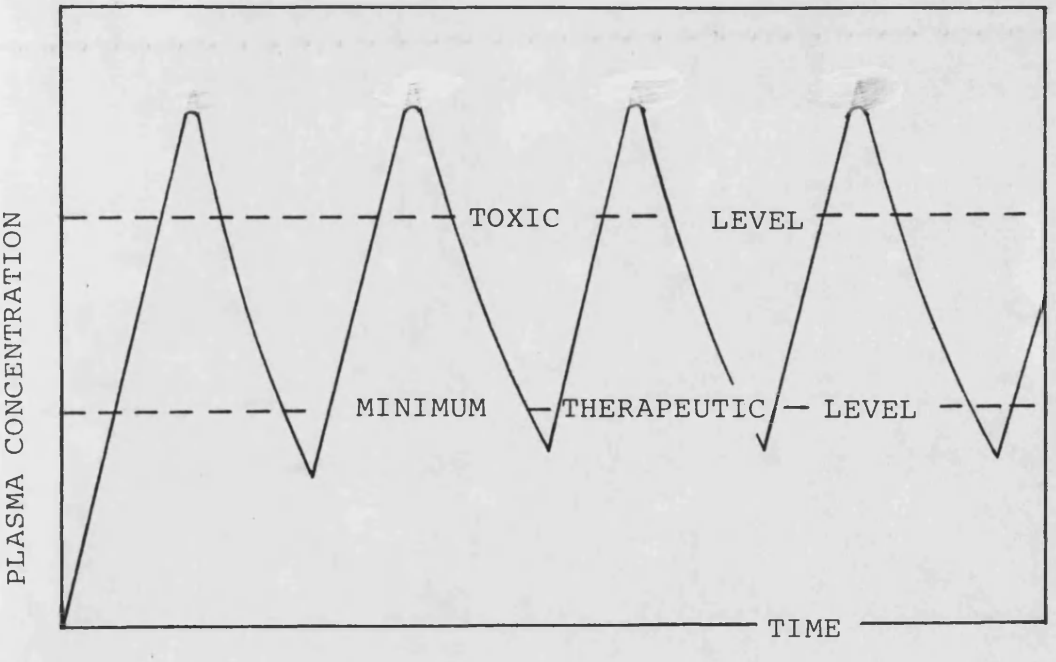



FIGURE 1.1b "PULSING" PLASMA CONCENTRATION PROFILE DURING REPEATED DOSING BY MOUTH

## 1.2. Routes of controlled drug administration

In conventional drug therapy, side-effects often arise from drug interactions with non-target tissues [13], so that locating a controlled drug delivery system in close proximity to the target organ may be beneficial. A number of devices adopt this principle; for example, a pilocarpine ocular system for the treatment of glaucoma ("Ocusert", Alza Corporation, California, U.S.A.), delivers drug to the eye for 1 week when placed under the lower eyelid [3]. An intrauterine progesterone delivery system for contraceptive use ("Progestasert", Alza Corporation) releases drug at a constant rate for at least one year [19]. An intraoral system for fluoride release has also been proposed [20]. Irritation caused by the Ocusert system has been reported [3] and disadvantages of Progestasert include bleeding, the risk of ectopic pregnancy as well as cervical and uterine perforations [19].

### 1.2.1. Drug targeting

Agents capable of locating at specific sites of action have been attached directly to drug molecules or to "drug carriers" such as liposomes, albumin microspheres and synthetic polymer nanoparticles [21,22]. In particular, monoclonal antibodies appear promising agents for the targeting of cytotoxic agents to malignant cells. However, even in cases where drug targeting agents are used, some form of controlled delivery may still be desirable.

### 1.2.2. Implants

Some of the earliest work using controlled release systems involved the subcutaneous implantation of silicone rubber-

encapsulated steroids [23]. Davis [24] attempted to control diabetes in rats using polyacrylamide implants which released insulin for periods of 2 to 3 weeks; animal weight was determined but blood glucose levels were not monitored. In similar work, Creque et al. [25] preferred ethylene-vinyl acetate copolymer to polyacrylamide on the basis of superior biocompatibility (section 1.3.5.) and demonstrated maintenance of normal blood glucose levels in diabetic rats for up to a month. However, a major disadvantage of implantable devices is the necessity for removal subsequent to useful drug release. Biodegradable polymers eliminate this problem, use of these materials has been reviewed by Wood [26]. Most work on biodegradable systems has been carried out in relation to long acting contraceptives. Biocompatibility of polymers is discussed below in section 1.3.5.

### 1.2.3. Oral administration and other routes

Because of the convenience of dosing by mouth, a number of sustained-release preparations for oral administration have been proposed. Koch-Weser and Schechter [27] were critical of oral sustained release devices suggesting that some have been created for commercial reasons only and that a reliably performing slow-release preparation is justified only when it increases the effectiveness and safety of the incorporated drug. A number of constraints are imposed by the oral administration route including the "first pass effect", drug stability in the gastro-intestinal tract, drug metabolism in the gut wall, gut motility and pH [28].

Various routes of drug administration which avoid the hepatic

first pass effect (and sometimes other constraints imposed by oral dosing) have been studied. For example, Morimoto et al. [29] considered vaginal insulin delivery to be promising whilst the nasal delivery of this hormone and other polypeptides has also been examined [30,31] but suffers from the problem of tissue irritation.

The rectal route has been widely studied for the administration of insulin [32], however emphasis has been directed towards enhancement of otherwise unacceptable absorption rates [33-37] and long-term continual drug administration via the rectum is of questionable value. Although drug absorption by the rectal route is generally regarded as avoiding the first pass effect, this may be dependent upon a number of factors including absorption site within the rectum and drug type [38].

### 1.2.4. Transdermal drug delivery

The use of skin as a portal for drug delivery has been widely studied because of its accessibility and rich blood supply. In order to design an efficient transdermal system and to optimise transdermal delivery of drugs, an understanding of skin structure and function is essential.

#### 1.2.4.1. Skin structure and function

In addition to its role as a container for body fluids and tissues, skin performs numerous other functions including that of a barrier to the entry of harmful or unwanted molecules from the external environment.

Human skin (figure 1.2.) comprises 2 distinct but interdependent layers of tissue: firstly, the outermost epidermis, which is

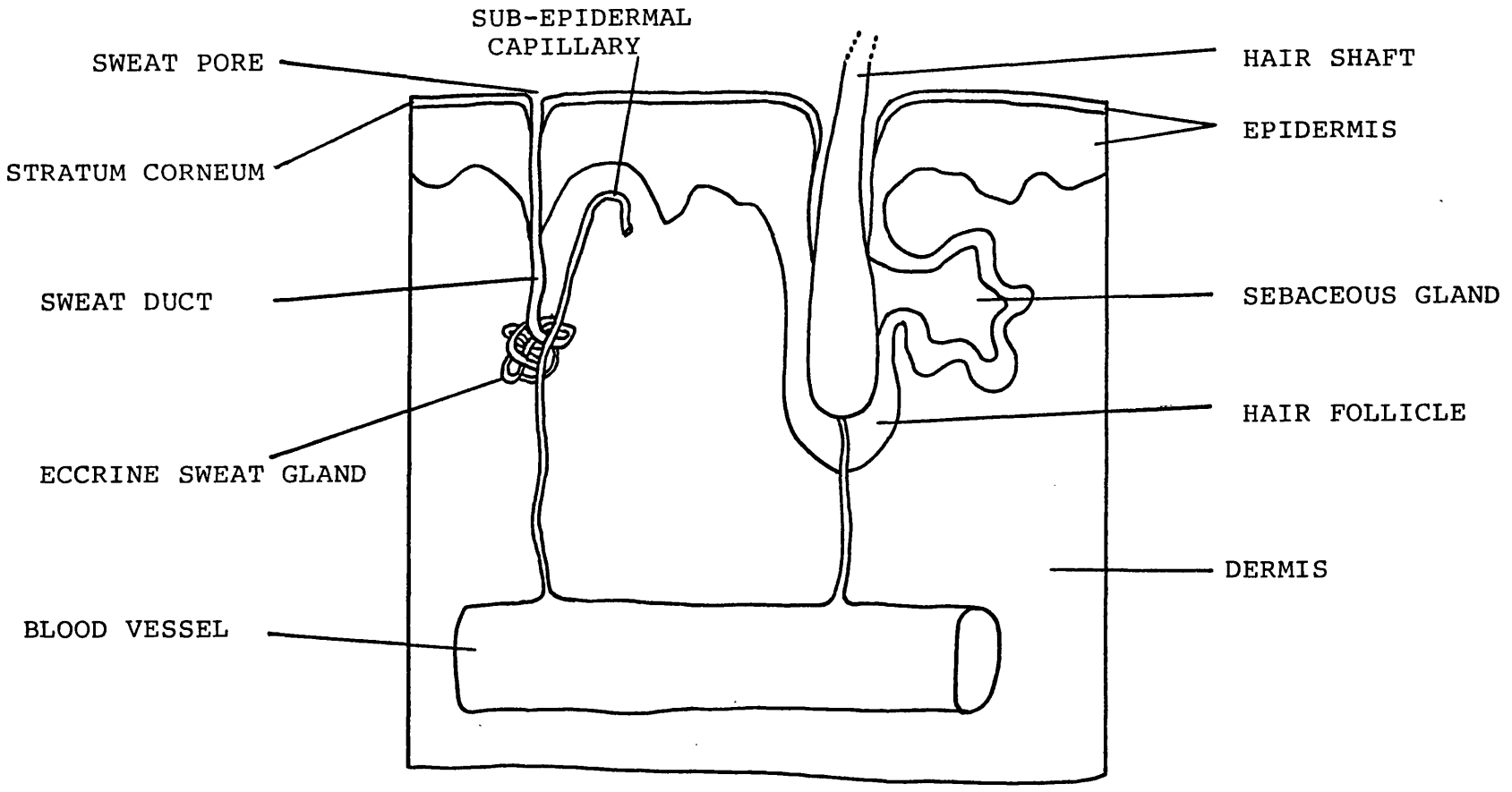



FIGURE 1.2 SCHEMATIC CROSS-SECTION OF HUMAN SKIN, MUCH SIMPLIFIED

unvascularised and approximately 100 um in thickness over most of the body with a range of 60 to 800 um according to anatomical location; and secondly, the dermis, which is 3-5 mm thick and interlaced with capillaries. Originating deep within the dermis and connecting with the external environment through the epidermis are hair follicles and sweat ducts [39].

### 1.2.4.1.1. Stratum corneum (horny layer)

The uppermost layer of the epidermis, the stratum corneum, is 10 to 50 um in thickness and composed of dead, partially desiccated, keratinised epidermal cells lying tangentially to the skin surface and stacked in highly organised vertical columns. In 1964, Blank [40] confirmed earlier postulations that the layer of cells dividing the stratum corneum from the remainder of the epidermis formed the principal resistance to the penetration of drugs. However, Scheuplein and co-workers showed that the horny layer itself was the location of molecular impermeability of skin and found the permeability of the entire epidermis to be indistinguishable from that of the stratum corneum alone [41,47]. Michaels et al. [39] proposed a simplistic 2-phase model of the stratum corneum which describes the tissue as a dispersion of hydrophilic protein in a continuous lipid matrix through which penetrant molecules migrate by dissolution and Fickian diffusion. Chandrasekaran found that experimentally determined *in vitro* permeabilities of several drugs through stratum corneum conformed to this model [42].

### 1.2.4.2. Factors influencing percutaneous drug absorption

It is usually assumed that foetal skin, and skin from elderly humans is more permeable than normal adult tissue [43,44] although Kligman [45] considered age to be unimportant in determining drug penetration.

Skin condition may be altered as a result of disease or injury when permeability generally increases [46].

Variation in drug absorption rate with anatomical site is well documented [47-49] and predictable according to the nature of the stratum corneum. Schleupin and Blank [47] ranked the diffusivity of simple, small molecules in increasing order of penetration: plantar, palmar and dorsum of hand, scrotal and postauricular, axillary and scalp, arm, leg and most permeable, the trunk. Shaw and Chandrasekaran [50] determined the transdermal flux of hyoscine hydrobromide in vitro using human skin and found increasing order of permeability to be thigh, forearm, stomach and chest, back and postauricular skin. They considered that the relatively high permeability of the skin behind the ear was due to several local morphological features including a relatively thin stratum corneum and a relative abundance of sweat and sebaceous glands. A transdermal system for the delivery of hyoscine hydrobromide uses postauricular skin as the site of application [42,50-54].

Drug may be metabolised within the skin prior to entering the circulation [55].

The normal water content in skin is approximately 40% (w/w) but this may be increased severalfold by surface occlusion or immersion in water. Hydrated stratum corneum is more permeable to virtually all molecules in comparison with skin in the normal state [47]; a

number of substances, e.g. urea [46], may enhance drug permeation by increasing skin hydration.

Drug binding in the skin may be significant, for example, in transdermal permeation of hyoscine hydrobromide a "dual sorption" model is employed and includes immobile drug molecules bound to skin sites and freely diffusing species [39]; mathematical analysis allows selection of an optimum priming dose of hyoscine hydrobromide to rapidly saturate immobilisation sites thus allowing steady state drug permeation to become established [42].

Since most drugs are either weak acids or bases, the degree of ionization will be dependent upon drug dissociation constant and solution pH [57]. Michaels et al. [39] have shown in vitro, using human skin, that hyoscine hydrobromide (pKa 7.35) in the unionized form is nearly 20 times more permeable than the corresponding ionized molecule.

Because of the lipid-protein nature of the stratum corneum, the drug oil/water partition coefficient is often used to predict drug skin-permeation behaviour. Highly water-soluble compounds having a strong tendency to partition into oils, generally show greatest permeation rates [39]; however, it is considered that interpretation of partition data may be complicated by drug binding and experimental conditions may relate poorly to in vivo use.

To increase the range of drugs available for transdermal delivery, agents causing a reversible reduction in barrier resistance properties of the skin have been studied. A number of such materials (usually termed "penetration enhancers") have been identified and these include: dimethylsulphoxide (DMSO), pyrrolidones, and surfactants, e.g.

1-dodecylazacycloheptan-2-one (Azone). Although the precise mechanism is unclear, most penetration enhancers operate by causing some physiological and/or chemical change in the stratum corneum. Barry [46] outlined properties desirable in an ideal penetration enhancer; most importantly, such agents should not cause severe irritation or damage to the skin.

### 1.2.4.3. Methods for the study of percutaneous absorption

Techniques used in percutaneous absorption studies have been reviewed by Nugent and Wood [60]. Wherever possible, human skin is preferred to animal tissue since physiological differences may lead to erroneous comparison; for example, Chowhan and Pritchard [61] found that neither rat nor rabbit skin was suitable as a model for the percutaneous absorption of naproxen through human skin.

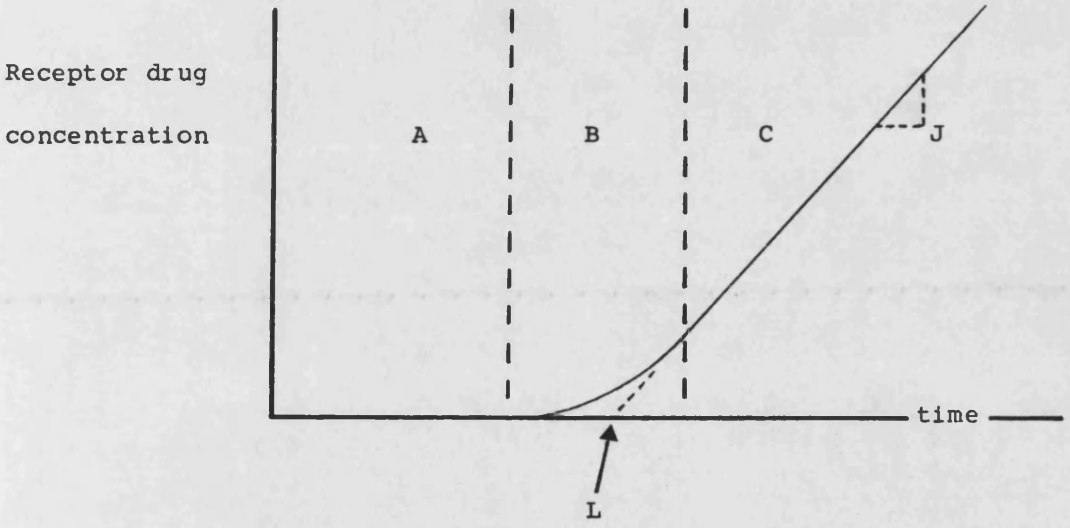
It is generally assumed that the stratum corneum provides the rate limiting step to percutaneous absorption and therefore most *in vitro* studies employ epidermis or stratum corneum alone rather than whole skin. Several techniques have been employed to separate epidermis from dermis and stratum corneum from epidermis, including heat separation and soaking in enzyme solutions [62]. Although Swarbrick et al. [63] have recently found that skin samples stored frozen show higher permeation rates than fresh tissue, most workers employ freezing for skin preparation and storage [39,61,62,215].

*In vitro* methods to study percutaneous absorption can be divided into infinite dose and finite dose techniques:

(i) **Infinite dose technique for studying percutaneous absorption of drugs**

In this method, skin is mounted as a barrier between two fluid-filled chambers which form the donor and receptor compartments. Permeation is assessed by periodic sampling of the receptor solution; however due to the high initial value, no appreciable reduction in donor concentration occurs since only a small fraction passes into the receptor, hence the term "infinite dose".

A typical release profile obtained in a permeation experiment using the infinite dose technique may be represented thus:




Three phases are apparent:

A. Lag phase when drug is not detected in receptor.

B. Non-linear phase when solute concentration in the receptor continuously increases.

C. Linear phase or steady state when flux (J) is constant and can be calculated from the slope of the linear portion using equation 1.1 [64].

$$J = \frac{dM}{dt} = \frac{DCo}{h} \qquad \text{(equation 1.1)}$$

where M is the mass of solute diffusing in time t

Co is the solute concentration in the first layer of membrane contacting the donor solution

h is the membrane thickness

D is the solute diffusion coefficient in the membrane

The lag time, L, may be obtained by extrapolation of the steady state plot to the time axis and may be used to calculate D using equation 1.2 [64].

$$L = \frac{h^2}{6D} \qquad \text{(equation 1.2)}$$

It is usually not Co but $Co^1$, (the initial concentration of solute in the donor solution bathing the membrane) which is measured. Co and $Co^1$ are related by K, the solute partition coefficient between the membrane and bathing solution (see equation 1.3).

$$Co = Co^1K \qquad \text{(equation 1.3)}$$

Substituting in equation 1.1 gives equation 1.4 [64]

$$\frac{dM}{dt} = \frac{DCo^1K}{h} \qquad \text{(equation 1.4)}$$

Because of difficulties often experienced in determination of K in biological membranes, the permeability coefficient, P (equation 1.5), a composite of K and D (see also section 4.4.2.) is often the parameter reported in the literature.

$$P = KD \qquad \text{(equation 1.5)}$$

(ii) **Finite dose technique for studying percutaneous absorption**

Franz [65] objected to the infinite dose technique mainly on the basis that both surfaces of the membrane are bathed by aqueous solution and thus fully hydrated skin is employed; to mimic in vivo conditions more closely a "finite dose" technique was proposed where the membrane was bathed only on one surface which was considered to model blood supply, whilst the other surface was exposed to a controlled environment and to which a given quantity of medicament was applied.

### 1.2.4.4. Transdermal drug delivery systems

The Alza Corporation has developed a number of "Transdermal Therapeutic Systems" (TTS) which resemble sticking plasters and consist of: an outer backing layer; a saturated drug reservoir; a diffusion rate-controlling membrane; and an adhesive, drug-containing layer contacting the skin surface (figure 1.3). TTS's designed to deliver glyceryl trinitrate, clonidine, hyoscine hydrobromide and oestradiol have been developed [52,66] and a number of other drugs, e.g. propranolol [67] have been studied for future systems. Transdermal systems avoid the hepatic first pass effect and also aim to lower drug dose and achieve constant plasma levels of drug. Pharmacokinetic models have been developed to describe release from transdermal rate-controlled devices [68,69].

Hyoscine hydrobromide has useful anti-emetic action but when administered orally or by injection at a dose of 200 ug every 6 hours, may produce unpleasant side effects such as drowsiness, giddiness, confusion and mental disturbances [53]. Transdermally, the TTS-hyoscine delivers approximately 140 ug of drug as a priming

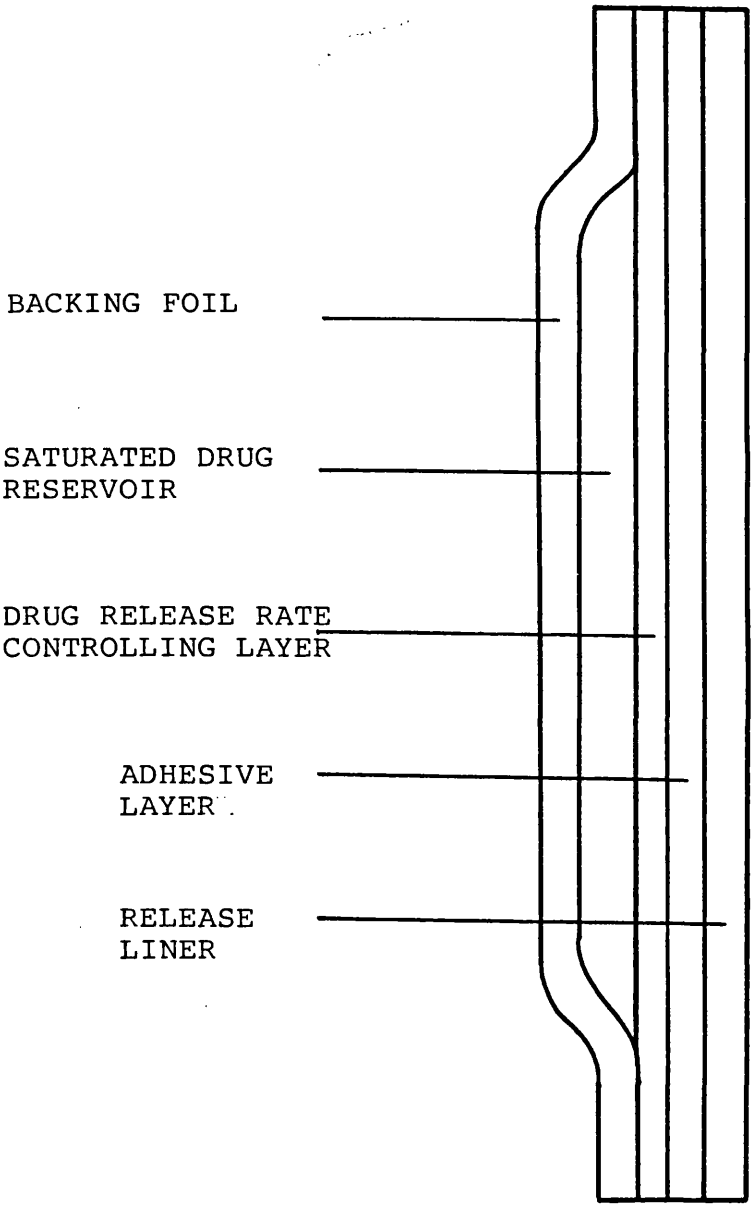



FIGURE 1.3 SCHEMATIC DRAWING OF A TRANSDERMAL THERAPEUTIC SYSTEM

dose in the first few hours to saturate drug binding sites within the skin followed thereafter by a constant delivery rate of 5 ug $hour^{-1}$ over the remainder of the 3 day treatment period. The system, of 2.5 $cm^2$ area is placed behind the ear; hyoscine release rate corresponds to 2 ug $cm^{-2}$ $hr^{-1}$ which is one fifth of the average permeation rate of the drug through postauricular skin [53], thus the system controls hyoscine permeation rate through the skin. The system provides protection against motion sickness with a reduction in the incidence and severity of side effects [51,70] associated with oral or intramuscular therapy.

Skin irritation produced by the prolonged application of transdermal therapeutic systems has been reported [71].

The penetration of drugs into skin by iontophoresis is discussed below in section 1.4.5.1.

## 1.3. Polymers used in sustained and controlled drug delivery systems

Synthetic polymers have been important in many of the proposed approaches to sustained and controlled drug delivery; a number of publications discuss the role of polymeric materials in this respect [3,5,13,17,72,73].

### 1.3.1. Preparation of polymers

Polymerization (and resulting polymers) can be divided into addition (vinyl) or condensation mechanisms according to the reaction stoichiometry [74]. Most polymers used in drug delivery devices are of the former type and comprise repeating monomer units forming a carbon backbone. Substituted ethylene generally forms the basis of vinyl polymers, substituent groups influencing polymer characteristics; preparation is usually by free radical polymerization involving a "chain reaction" where monomers add only to molecules containing active centres, the monomer concentration steadily decreasing as the polymer chain grows [73].

The condensation polymer backbone usually comprises more than one element; these materials account for a small fraction of all synthetic polymers and have been less frequently used in drug delivery systems.

Polymerization reactions can be further classified according to the processing technique employed, into either homogeneous or heterogeneous methods [73]. The former includes bulk polymerization involving only the monomer and initiator, as well as solution polymerization where reaction occurs in solvent. Heterogeneous systems include emulsion and suspension polymerization methods.

### 1.3.2. Copolymers

Copolymers are composed of more than one type of monomer unit (m) and can be produced by addition or condensation polymerization. There are 4 main classes of copolymer:

(a) alternating copolymers, with a regular order of monomer species in the polymer chain;

(b) random copolymers where sequences of $m_1$ and $m_2$ are arranged indiscriminately;

(c) block copolymers contain long sequences of identical repeating units, i.e. $(m_1)n(m_2)n$

(d) graft copolymers, in which chain extensions of a second monomer occur as branches.

Ethylene-vinyl acetate (EVAc) copolymer and lactic acid-glycolic acid copolymer are of the random variety and have been studied for use in drug delivery systems [75-77].

### 1.3.3. Crosslinked polymers

3-dimensional networks can be formed by the incorporation of a bifunctional agent to crosslink polymer chains during polymerization. Hydrophobic crosslinked polymers such as silicone rubber were amongst the first materials used in controlled release devices [23]. In 1960, Wichterle and Lim [78] first proposed hydrophilic gels or "hydrogels" e.g. poly(2-hydroxyethyl methacrylate) or polyHEMA for prosthetic implantation and contact lenses.

Hydrogels are materials which swell in water and other fluids by absorbing liquid without dissolving. A number of hydrogels have been proposed for use in controlled drug delivery systems [7,72,79,83];

the most common synthetic hydrogels studied as potential biomaterials are polyHEMA, poly(methacrylamide), poly(vinyl alcohol), poly(N-vinyl pyrrolidone) and polyacrylamide. Polyacrylamide is also employed as a supporting medium in the electrophoresis of proteins (section 1.5.3.1.).

### 1.3.4. Characterization of polymers

Molecular weight determination, thermal analysis and mechanical testing of polymers are methods routinely employed in their characterization [84,]. Evaluation techniques relating more directly to the usage of polymers in controlled drug delivery systems include solute diffusion through polymers [85,86], swelling of hydrogels and polymer biocompatibility (see also sections 1.3.5. and 1.4.1.4.c.).

### 1.3.5. Biocompatibility of polymers

A biocompatible polymer may be defined as a material which does not adversely affect its biological environment by producing undesirable effects such as tissue irritation or more seriously, tumours. Clearly, biocompatibility is most important in materials for implantation.

Several hydrogels, e.g. poly(HEMA), polyacrylamide and poly(glycol monomethacrylate) have been shown to possess good biocompatibility characteristics [87-89]. Variations in material purity can make comparison of biocompatibility between polymers difficult. For example, Langer and Folkman [90] implanted various polymers in the eyes of rabbits and found that EVAc caused no inflammation whilst polyacrylamide was significantly inflammatory, however only the former had been subjected to an exhaustive washing

procedure.

Bruck [89] considered not only polymer hydrophilicity to be of importance in determining biocompatibility but also believed water structure both within the gel and on its surface to be important.

Water in hydrogels is believed to exist in at least 3 distinct forms [91,92]: bound water, which is hydrogen bounded to the polymer chains; bulk or normal water, which has the properties of solvent water; and interfacial water, an intermediate form.

Sprincl et al. [88] suggested that hydrogel physical form may affect its biocompatibility, they found that homogeneous gels showed superior tissue compatibility to heterogeneous or "phase separated" gels. Thus not all factors affecting polymer biocompatibility are understood.

## 1.4. Energy sources for producing controlled drug delivery

A controlled drug delivery system operates by the controlled use of an energy source to effect drug release. Energy sources which can be used in drug delivery devices include: physicochemical (e.g. diffusion, osmosis, dissolution and biodegradation), mechanical, electrical and magnetic energy.

### 1.4.1. Diffusion (in polymers)

To date, diffusion has been the main energy source employed in polymeric drug delivery systems and may be described as the tendency for gases or solutes to spread uniformly throughout a medium under the driving force of a concentration gradient; diffusion is a direct result of Brownian motion. Barrer [93] considered the diffusion phenomenon in polymers to result from changes in thermal energy within the polymer which when sufficiently high, allow the diffusing molecule to move between the polymer chains. The "free volume" theory of Kumins and Kwei [94] states that fluctuating local density in the polymer creates free volume which if large enough, allows the diffusant to move into it. With both theories, the passage of a large molecule through a polymer would be expected to be slower than for a small molecule.

By analogy to heat conduction, Fick's first law of diffusion relates to the mass transfer rate or flux, J, of a solute across a plane of area A (equation 1.6).

$$\frac{dM}{dt} = J = -DA \frac{dc}{dx} \qquad \text{(equation 1.6)}$$

where $M$ is the mass of solute diffusing in time $t$

$D$ is the diffusion coefficient for the solute in the system.

$\frac{dc}{dx}$ is the concentration gradient of the solute across the diffusion path.

A negative sign is used in equation 1.6 since diffusion is from a high concentration to a low concentration region (until an equilibrium state is reached).

Variation in diffusion coefficients of solutes through polymers is marked and dependent upon a number of factors, e.g., temperature, polymer state, solute molecular weight and size.

The release of solute from polymeric drug delivery systems can be divided into 2 main types and it is appropriate to consider (i) drug diffusion from matrices and (ii) drug diffusion across membranes (i.e., from reservoir devices).

### 1.4.1.1. Diffusion across membranes

The majority of reservoir drug delivery systems comprise a saturated drug solution or suspension enclosed by a polymeric membrane; the undissolved drug acts as a constant source of solute available for diffusion. If sink conditions prevail and there are no diffusion effects in the boundary layer, then drug release per unit surface area from a capsular reservoir may be defined by equation 1.7 [95].

$$J = \frac{dM}{dt} = \frac{D\ Cs}{x} \qquad \text{(equation 1.7)}$$

where Cs is the solubility of drug in the polymer

x is the membrane thickness

It can be seen from equation 1.7 that the release rate is a linear function of both drug solubility in the polymer and membrane diffusivity and inversely proportional to membrane thickness and exhibits zero-order kinetics over the useful lifetime of the device. Modifications to equation 1.7 may be required according to specific system characteristics.

Initial lag or burst effects may be observed if the system is used before a concentration gradient is established within the membrane or if saturation of the membrane occurs during storage [96]. After some time, release rate will fall since the mass of solute per unit volume in the core will approach the solubility of the drug.

A number of systems utilising a membrane to control drug release have been proposed and many of these are currently in use: several intrauterine controlled delivery devices release contraceptive hormone e.g. Progestasert [19]; the Ocusert therapeutic system [3] also uses ethylene-vinyl acetate copolymer to control drug diffusion thereby delaying the delivery of pilocarpine to the eye for the treatment of glaucoma; drug reservoir devices in the micron size range (microcapsules) have been proposed for the release of pilocarpine for ocular therapy [97]; reservoir devices for transdermal use are discussed above in section 1.2.4.4.

### 1.4.1.2 Diffusion from matrices

Matrix devices usually comprise a dispersion of drug particles or crystals within an inert polymer matrix. Higuchi [98] developed equation 1.8 to describe solute release from a planar system having a homogeneous matrix.

$$Q = (D\ t\ (2T - Cs)Cs)^{0.5} \qquad \text{(equation 1.8)}$$

where Q is the amount of drug released per unit area after time t

T is the total amount of drug in the polymer matrix

Higuchi suggests that the model would be suitable only where T exceeds Cs by a factor of 3 or 4, i.e., when most of the drug is present as solid or dispersed particles, in which case equation 1.8 reduces to equation 1.9.

$$Q = (2Cs\ D\ T\ t)^{0.5} \qquad \text{(equation 1.9)}$$

Dispersed drug particles must first dissolve in and then diffuse through the polymer network before being eluted from the matrix surface. A depletion zone arises at the surface and becomes thicker as elution continues towards the centre of the matrix. Thus the diffusion path length increases with time and drug release becomes time dependent; a linear $Q - t^{0.5}$ relationship is observed.

Controlled release matrix devices have been proposed for the delivery of a number of drugs, e.g., steroids from vaginal rings [99] and from silicone polymer matrices [100]. A number of workers have

attempted to achieve zero-order release kinetics from matrix drug delivery devices by variation of matrix geometry and use of impermeable coatings [75,101].

### 1.4.1.3. Diffusion from porous matrices

Capillaries or channels filled with solute particles may result if a sufficient drug loading is attained [12,73]; in an inert hydrophobic matrix, drug permeation through the polymer is negligible and occurs instead by dissolution and diffusion through the channels and pores following the influx of water (e.g. body fluids). Equation 1.10 was developed by Higuchi [98] to describe release from porous matrix tablets.

$$Q = \left(\frac{DaE(2T-ECa)Cat}{r}\right)^{0.5} \qquad \text{(equation 1.10)}$$

where Ca is the solubility of the drug in the permeating fluid.

Da is the diffusion coefficient of the drug in the permeating fluid.

E is the polymer porosity factor.

r is the polymer tortuosity factor.

The terms E and r are used to describe the channels and pores in individual systems.

Porous matrices have been developed for administration of drugs by the oral route using plastic matrix tablets [102] and by implantation using EVAc [103].

### 1.4.1.4. Diffusion in hydrogels

The hydrophobic polymers described in sections 1.4.1.1. to 1.4.1.3. have minimal interaction with water, whereas hydrogels have a significant aqueous content. Diffusion in gels was recently reviewed by Muhr and Blanshard [104] and covered a spectrum of gels ranging from one-phase solutions where "pores" are neither constant in size or location, to two-phase or heterogeneous rigid-porous structures; release profiles from the latter type being similar to those obtained from porous devices (see section 1.4.1.3.).

A number of factors affect solute diffusion in gels [104] and these include: obstruction by polymer molecules; hydrodynamic drag at the polymer-solvent interface; alteration of solvent (water) properties by the polymer; and polymer-solute interactions such as binding.

Hydrogels are permeable to hydrophobic as well as hydrophilic solutes, and drug transport can occur by a "pore mechanism" whereby diffusion occurs through bulk water within the pore network or by a "partition mechanism" [82] similar to that described above (section 1.4.1.2.) involving dissolution and diffusion in the polymer. Several factors affecting solute diffusion in hydrogels are discussed below:

(a) **Hydrogel water content and solute diffusion**

Davis [86] developed an equation which can be used to estimate the diffusion coefficient of a solute in a hydrogel from its diffusion coefficient in water (equation 1.11).

$$D = Do \exp(-(0.05 + 10^{-6}M)P) \qquad \text{(equation 1.11)}$$

where D is the diffusion coefficient of solute in polymer

Do is the diffusion coefficient of solute in water

M is the molecular weight of the solute

P is the percentage of polymer in gel

Yasuda et al. [105] have also shown that diffusion through hydrogels, e.g. poly(HEMA), will increase with increasing hydration.

The pore mechanism predominates in strongly hydrated hydrogels whereas the partition process plays an increasingly important role as the degree of hydration decreases [83] and becomes even more significant for larger solutes [80].

(b) **Crosslinking in hydrogels and solute diffusion**

Increased crosslinkage in hydrogels results in a reduced pore size and a decreased fraction of bulk water; consequently solute diffusion coefficients decrease and the partition mechanism may dominate the permeation process [106-108].

(c) **Hydrogel swelling and solute diffusion**

A problem which is characteristic of diffusion in gels is that the gel volume may not remain constant because the gel has not reached an equilibrium degree of swelling, indeed swelling may be caused by the progress of solute diffusion. The drug release rate is modified by the extent of hydration of the drug-polymer device, and the nature and concentration of the drug modifies the rate and extent of hydration.

A hydrogel is capable of imbibing water and simultaneously releasing enclosed drug, the diffusion of solute may be slowed by a countercurrent caused by water uptake by the hydrogel [83,110].

Complete mathematical analysis of drug release from swelling hydrogels is difficult because diffusion is accompanied by a change of phase with a moving boundary separating the phases. Lee [109] solved certain moving boundary problems encountered during solute release from hydrogels and obtained approximate solutions which compared well with available exact solutions. A square root of time-dependency for drug release rate is predicted by a model derived by Peppas et al. [110] which considers swellable hydrogels exhibiting significant volume expansion but with constant drug diffusion coefficients. Good [83] described a mathematical model employing time-dependent drug diffusion coefficients with continuous swelling but at a constant total volume.

### 1.4.2. Osmosis

Separation of a solution from pure solvent by a semipermeable membrane (allowing only the permeation of solvent molecules) leads to the phenomenon of osmosis where solvent passes spontaneously into the solution so that an osmotic pressure is set up [111].

Probably the simplest osmotic delivery system was first described by Theeuwes and Higuchi in 1974 ("OROS") and comprises a drug reservoir enclosed by a semipermeable shell [112]; the device outwardly resembles a tablet. After administration, an osmotic pressure is created within the device by the influx of water from the gastro-intestinal fluids and drug solution is expelled through a laser-drilled aperture in the wall of the device.

OROS-indomethacin was the first oral osmotic system introduced in Europe but was quickly withdrawn due to problems caused by adherence of the device to the gut wall leading to localised drug release which produced cases of peptic ulceration with bleeding and perforation [113]. Smith et al. have proposed a device with several orifices for osmotic drug delivery [114] which may overcome some of the problems of highly localised drug release. Recently, Breimer et al. [115] studied osmotically controlled rectal delivery of several drugs, but concluded that therapy would be limited to treatments of relatively short duration.

Virtually all diffusion and osmotically controlled drug delivery systems are capable at best of achieving zero-order release kinetics, usually according to predetermined rates. Some energy sources which may be controlled to provide more variable drug release rates (as may be required in certain disease states) are discussed below in sections 1.4.3 to 1.4.5.

### 1.4.3 Magnetic energy

Langer and co-workers have reported the use of oscillating magnetic fields to regulate drug release from polymer matrices [3,12,77,116-118].

Initially, bovine serum albumin (BSA) and magnetic steel beads (1.4 mm in diameter) were incorporated in an ethylene-vinyl acetate copolymer (EVAc) matrix in the form of a slab [3,116]. When exposed to an aqueous medium, BSA was slowly and continuously released from the polymer by diffusion; application of an oscillating magnetic field increasing the release rate by as much as 100%. However, BSA release rate was found to decrease with time due to matrix geometry (see section 1.4.1.2.). The use of a coated hemispheric system [117] containing a magnetic ring inside the polymer matrix achieved a constant baseline level release rate with magnetically modulated bursts of BSA release on demand.

It has been speculated that the oscillating magnetic field causes alternate compression and expansion of drug-containing pores thus causing increased release [116] and may also affect the macromolecules in some way [77]. Although video recordings have shown motion of the polymer material on application of the magnetic field [77], the precise mechanism of magnetic action is unclear.

### 1.4.4. Mechanical energy

Controlled drug delivery systems utilising mechanical energy are usually infusion pumps which generally rely on electrical energy for their operation. Those systems using electrical energy more directly to control drug delivery are discussed below in section 1.4.5.

The simplest pumps provide continuous and constant-rate drug infusion. For example, Boulton et al. [120] found improvement (over conventional therapy) in patients with painful diabetic neuropathy when treated by continuous subcutaneous infusion of insulin.

Several feedback-controlled infusion systems have been developed, e.g. "BIOSTAR" developed by Miles Laboratories, Indiana, USA [121] is a computerised system designed to simulate the function of the normal pancreas in the regulation of blood glucose levels; insulin infusion rate is governed by results of frequent blood analyses. A drawback with BIOSTAR and similar systems is their size, making patient mobility difficult, thus much effort is currently being devoted to the development of implantable drug (especially insulin) delivery systems [122,123,124].

Schade et al. [122] listed desirable features of an implantable insulin delivery system including (a) reliability (b) easy implantation and removal (c) long battery life (d) multiple insulin delivery rates (e) small size and weight and (f) biocompatibility.

Buchwald et al. [124] overcame the problem of drug depletion in implanted devices for the treatment of deep venous thrombosis by refilling pump reservoirs by percutaneous injection of heparin, however episodes of pump refill-associated haemmorrhage were found.

Some workers such as Sefton et al. [123] consider that currently available glucose sensors are inadequate for feedback control and favour "open loop" or preprogrammed insulin delivery.

### 1.4.5 Electrical energy

Mechanical pumps (section 1.4.4.) are probably the largest users of electricity for the purposes of achieving controlled drug delivery. An important alternative use of the above energy source is for iontophoretic drug delivery:

#### 1.4.5.1 Iontophoresis

The term iontophoresis has been described as "the transfer of ions under electrical pressure into the body surface for therapeutic purposes" [125]. Charged drug is applied under an electrode of the same polarity; a return electrode, opposite in charge to the drug, is placed at another body site and current (below pain threshold levels) allowed to flow for an appropriate time [126].

The delivery of steroids, local anaesthetics and a beta-blocking drug by iontophoresis has been investigated with promising results [127-129]. Gangarosa and Park [125] have reviewed the use of fluoride iontophoresis for desensitising dentin.

Drugs for administration by iontophoresis must be charged and of low molecular weight [128,130]; high molecular weight molecules e.g. insulin (m.w approximately 5800) are currently found to be inapplicable by iontophoresis [217].

A bracelet-drug delivery device [130] which works on the principle of iontophoresis has been developed ("Panoderm", Elan Corporation, Ireland).

Iontophoresis differs from electrophoresis in that the former term implies passage through the skin while electrophoresis is performed in a number of media (section 1.5.3.1). Electrophoresis has not previously been studied as a means of providing controlled drug delivery.

## 1.5. Electrophoresis

### 1.5.1. Electrolysis and ionic migration

When a current is passed through a solution of an electrolyte, the ions move towards the electrodes. In a medium where electronic conduction does not occur, the whole current may be carried by ions. Ionic migration during electrolysis is often called ionophoresis [131] and is analogous to electrophoresis which involves the movement of charged coarse or colloidal particles in liquid under the influence of an applied electric field.

McDonald [56] discussed terminology used to describe the electromigration of all types of migrants (e.g. colloidal materials such as proteins as well as ionic species) in stabilized media; it was considered that nomenclature was considerably confused.

During this study, migration of colloidal and molecular species under the influence of an applied electric field is referred to as "electrophoresis" if performed using a stabilized electrolyte medium such as polyacrylamide gel.

### 1.5.2. Electrokinetic phenomena [132,133]

Electrophoresis is one of 4 related electrokinetic phenomena: Electrophoresis involves the motion of a charged surface relative to stationary liquid under the influence of an applied electric field; electro-osmosis, essentially the opposite of electrophoresis, is the movement of liquid relative to a stationary charged surface; the sedimentation potential is that created by the movement of charged particles relative to stationary liquid; and the streaming potential, which is caused by the flow of liquid along a stationary charged surface. These phenomena arise due to the nature of the solid-liquid

interface.

### 1.5.2.1. Surface charge and the electric double layer [132,133,134]

A material, dissolved or suspended in a polar medium (such as an aqueous solution of electrolyte) may acquire a surface charge by ionization or ion adsorption. For example, biological macromolecules such as proteins are dispersed in solution as particles of colloidal size. These possess a certain electrical charge due to groups capable of dissociating electrolytically. The net charge of a protein is determined by the pH of its medium, and may be modified by interaction with small molecular weight ions or other macromolecules.

Oppositely charged counter-ions are attracted to the particle surface by electrical forces whilst co-ions (of like charge) are repelled from the surface. By a combination of electrostatic forces and thermal mixing, an equilibrium situation results where excess counter-ions approach the surface and the remainder are distributed in decreasing amounts as distance from the surface increases until electrical neutrality prevails.

The electrical distribution at the interface is equivalent to a so called "diffuse double layer" of charge (figure 1.4.), the first layer being tightly bound and the second which is more diffuse. (The diffuse layer of countercharges occurring in ionophoresis is usually referred to as an "ionic atmosphere" [131]). The degree of attraction between the tightly bound layer of counter-ions (in the region bounded by AA' and BB') and the surface is such that the movement of the surface relative to liquid results in BB' being the shear plane rather than the true surface, AA' (figure 1.4). The

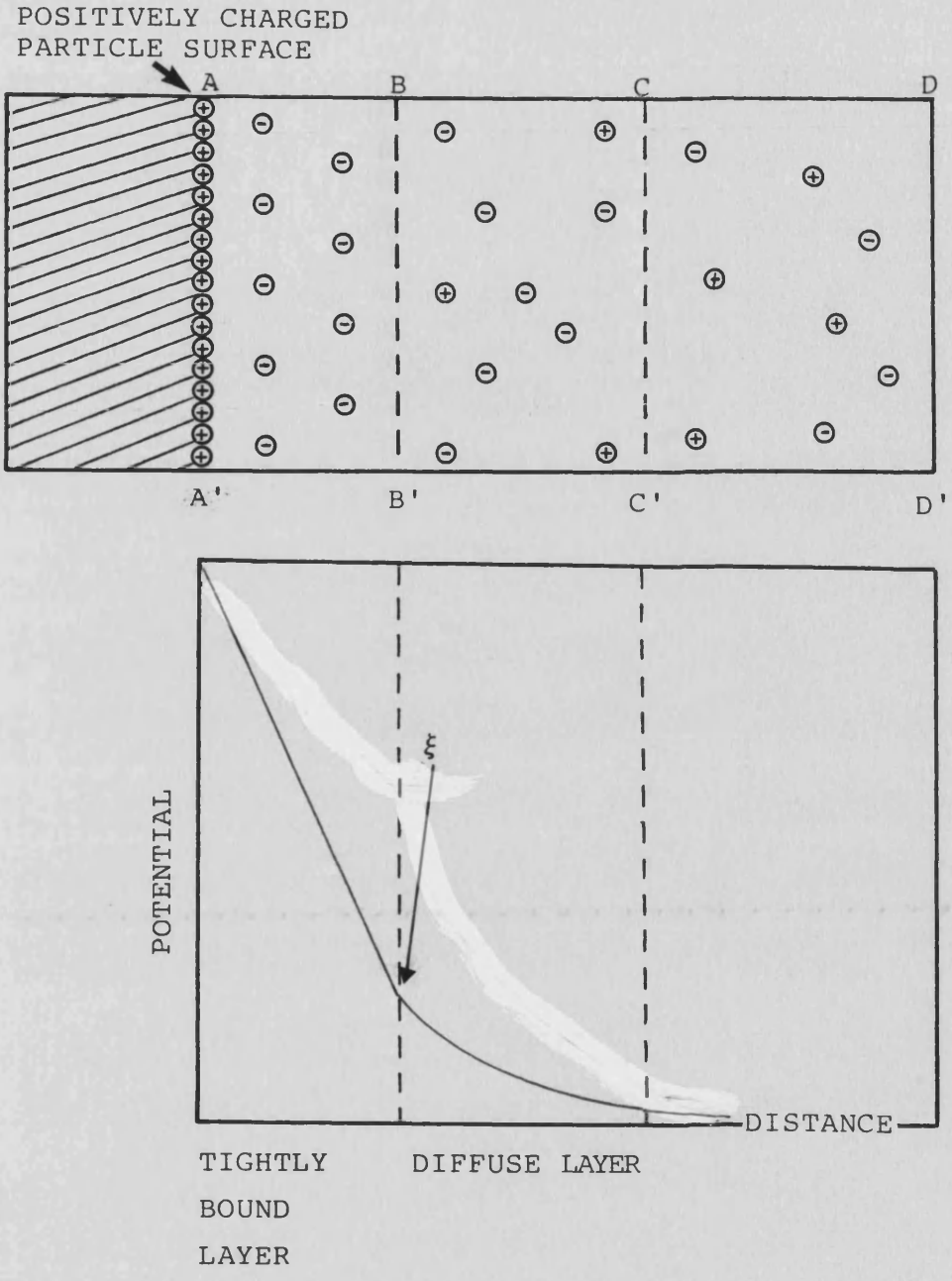



FIGURE 1.4 THE ELECTRICAL DOUBLE LAYER AND THE ELECTROKINETIC POTENTIAL AT THE SOLID-LIQUID INTERFACE

potential decreases as distance from the surface increases and at the solid surface, AA' (with respect to the electroneutral region of the solution) is termed the Nernst potential, the lower potential at the shear plane BB' is the electrokinetic or zeta potential, $\xi$ , (figure 1.4.). The potential decreases in this manner because counter-ions close to the surface act as a screen thus reducing electrostatic attraction between the charged surface and counter-ions further away from the surface. In the regional BB' to CC', there is still an excess of counterions, beyound CC', the distribution of ions is uniform and electrical neutrality is obtained.

Increasing the electrolyte concentration or counter-ion valency causes the screening effect to increase, resulting in a more rapid decrease in potential since the double layer thickness is reduced. Depending on the nature of the particle surface and surrounding ions, electric potential-distance profiles differing from that shown in figure 1.4 may be obtained.

### 1.5.2.2. Zeta potential and electrophoretic mobility

Electrokinetic behaviour depends upon the potential at the plane of shear ($\xi$). This is located a small distance further out from the surface of the tightly bound layer, or the Stern plane, but the two appear experimentally indistinguishable and are generally assumed to be identical [131]. Zeta potential is important in the study of colloidal dispersions. Since it is the electrophoretic migration rate-determining potential, a number of electrophoretic techniques have been used to determine zeta potential [131,133].

Consider an isolated fluid-suspended particle with a net charge, Q; application of a uniform electric field, E, causes an

electromotive force, QE, to act on the particle. When the particle is set in motion from rest, a frictional force, fv acts in the opposite direction where v is the particle velocity and f is the frictional coefficient. For spherical particles, f is $6\pi na$, according to Stokes' law, where n is the solvent viscosity coefficient and a is the particle radius. The electrophoretic mobility of the particle, U, is defined by equation 1.12 and its sign is the same as the net charge of the migrating particle [136].

$$U = \frac{v}{E} \qquad \text{(equation 1.12)}$$

The relation of electrophoretic mobility to the zeta potential is discussed below:

Hückel [137] approached the problem of electrophoretic mobility of a sol particle in an electric field by considering a small sphere as a point charge but large enough for Stokes' law to be applicable. The total force acting on the unit was taken as the sum of viscous drag and electric force. Electrophoretic mobility is given by equation 1.13.

$$U = \frac{v}{E} = \frac{Q}{6\pi na} \qquad \text{(equation 1.13)}$$

The zeta potential, $\xi$, at the surface of shear is given by equation 1.14 where c is the permittivity of the electrolyte medium; ka is a

$$\xi = \frac{Q}{ca(1+ka)} \qquad \text{(equation 1.14)}$$

dimensionless quantity which is the ratio of radius of curvature to double layer thickness [132]. For a point charge, ka can be neglected, giving the Hückel equation (1.15) for electrophoretic mobility.

$$U = \frac{\xi c}{6\pi n} \qquad \text{(equation 1.15)}$$

In a second approach, Smoluchowski [138] considered the motion of liquid in the diffuse part of the double layer relative to that of a non-conducting flat surface of a particle whose radius is large and double layer thickness small (i.e. large ka). Application of an electric field parallel to the surface exerts an electric force which is the product of charge and field strength (the assumption was made that the decrease in potential with distance from the particle surface is governed by the Poisson equation). A viscous force will also be exerted and will be proportional to the difference in shear rate over the thickness of the liquid layer. By equating the 2 forces and assuming that permittivity and viscosity are constant throughout the double layer, the Smoluchowski equation for electrophoretic mobility is obtained (equation 1.16).

$$U = \frac{\xi c}{4\pi n} \qquad \text{(equation 1.16)}$$

Equations 1.15 and 1.16 relate electrophoretic mobility to zeta potential but differ by a factor of 4/6; one applies to small ka (<1) and the other to large ka (>100). These equations were developed for non-conducting rigid spheres moving in a perfect insulator and are

inappropriate to electrophoresis of macromolecules which is performed in electrolytic solutions and gels [136]. In addition, account must be taken of the ionic atmosphere around the migrating particle which leads to relaxation and retardation effects (section 1.5.2.3).

### 1.5.2.3. Electrophoretic retardation and relaxation

The presence of an ionic atmosphere around a particle results in slower electrophoretic mobilities than predicted by equations 1.15 and 1.16. Local movement of liquid caused by a net movement (in the opposite direction to that of the particle) of ions in the mobile part of the double layer opposes the particle motion, an effect known as electrophoretic retardation. Electrophoretic relaxation arises from the distortion of the ionic atmosphere during electrophoresis, leading to a net force reducing the particle velocity [134].

Henry [139] derived a more general electrophoretic equation for conducting and non-conducting spheres in a conducting solution, allowing for the retardation effect but not accounting for electrophoretic relaxation. By assuming that the zeta potential is small, the Henry equation can be written as (equation 1.17):

$$U = \frac{\xi c}{6\pi n} f(ka) \qquad \text{(equation 1.17)}$$

where f(ka) varies between 1.0 for small ka (Hückel equation) and 1.5 for large ka (Smoluchowski equation). Henry also assumed that c and n were constant throughout the mobile part of the double layer.

The mobility (U) of a particle is a physical constant under defined electrophoretic conditions [135]. It can be seen from equation 1.12 that the absolute mobility is obtained by dividing the migration velocity, v, by the applied electric field, E. Electrophoretic equations as described above have application mainly in the determination of electrokinetic potentials. Absolute mobilities and zeta potentials are rarely used in the electrophoresis of proteins in polyacrylamide gels since such studies are usually comparative exercises where the term "relative mobility" is preferred.

### 1.5.3. Applications of electrophoresis

Electrophoresis finds application in studies of colloid stability and ion-adsorption where calculation of zeta potentials, is involved. Electrophoresis is also widely used for the separation and identification of components of protein mixtures.

#### 1.5.3.1. Electrophoresis of proteins

Proteins are ampholytes whose charge is determined by the pH of the medium and the number and character of ionizable (amino and carboxyl) groups. A protein has zero net charge at its isoelectric point (pI) and will not migrate in an electric field.

In the 1930's Tiselius [140] pioneered electrophoretic analysis of protein mixtures, his "moving boundary" method comprised a glass U-tube in which dissolved protein mixtures were separated and identified. Temperature control was essential to minimise convectional boundary disturbances arising from the heating effect of the applied current.

Paper electrophoresis [141] became widely used in the 1950's with the advantage over Tiselius' method of providing improved separation of components of protein mixtures; apparatus normally comprising, a paper strip or sheet held between 2 electrolyte tanks. The disadvantages of this technique include protein adsorption onto the paper, fluid evaporation due to ohmic heating leading to increased ionic strengths and electro-osmosis. The latter phenomenon occurs because cellulose matrices are generally negatively charged [142] and induce a positive charge in contacting water which therefore moves under a potential gradient (section 1.5.2.) thus

influencing the sample mobilities.

Although silica gels had previously been employed as electrophoretic media [143], significant improvements in resolution were obtained by Smithies [144] using starch gels and later by Raymond and Weintraub [145] with synthetic polyacrylamide gels.

Starch, and in particular, polyacrylamide gels minimised convection and diffusion disturbances with improved resolution attributable to their "molecular sieving" effect (section 1.5.4.1.1.).

### 1.5.4. Polyacrylamide gel electrophoresis (PAGE)

The use of PAGE for the separation of proteins and nucleic acids is now widespread. Protein adsorption and electro-osmosis effects are negligible and a range of gels can be manufactured to meet specific requirements. There have been numerous publications related to PAGE, relevant bibliography is referred to in the following text.

#### 1.5.4.1. Formation and structure of polyacrylamide gel

Polyacrylamide gel is formed by the free radical polymerization of acrylamide monomers into long polyacrylamide chains. The elongating polymer chains are randomly crosslinked with N,N'-methylene-bis-acrylamide (Bis) resulting in a 3-dimensional network structure (figure 1.5) which behaves as a hydrogel (section 1.3.3). A number of parameters are important in polymerization of polyacrylamide gels:

(a) **Purity of gel-forming reagents**

Good quality reagents are essential since contaminants may

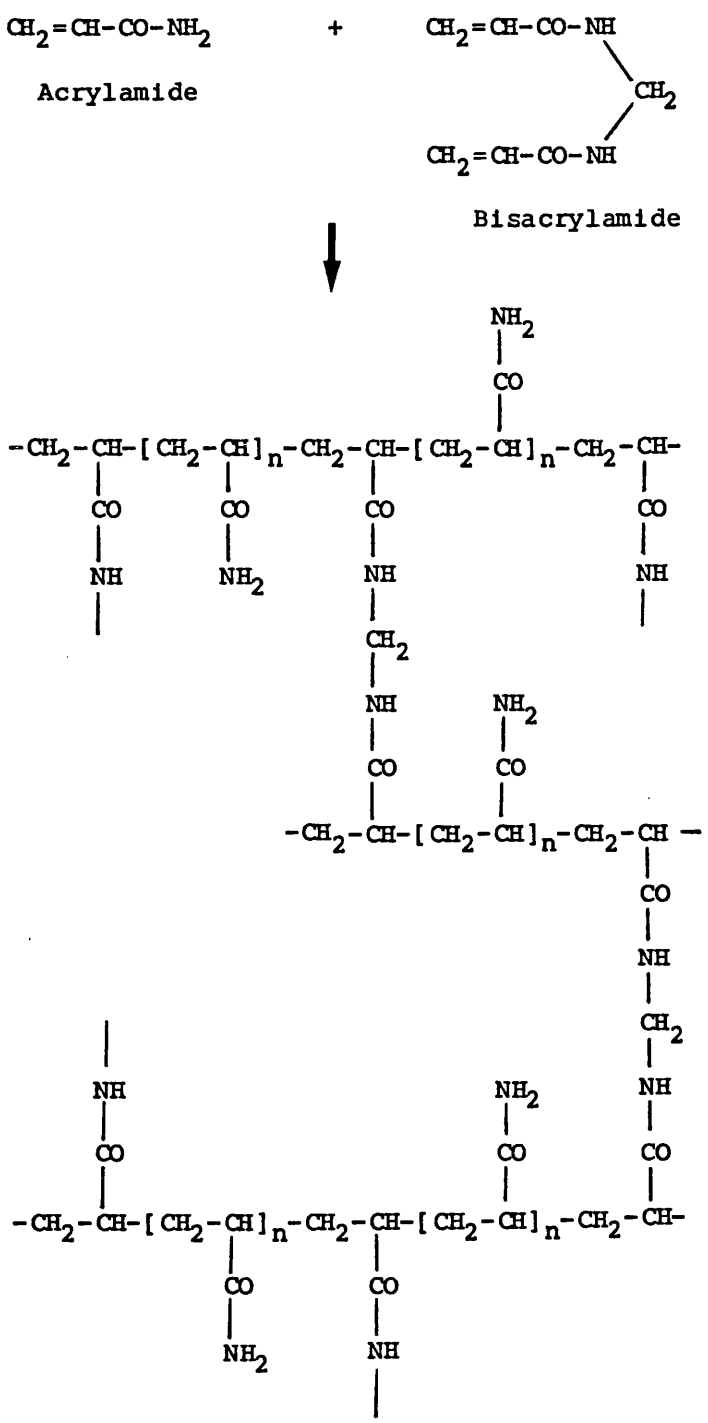



**FIGURE 1.5 STRUCTURE OF POLYACRYLAMIDE GEL MATRIX FORMED BY COPOLYMERIZATION OF ACRYLAMIDE MONOMER AND BISACRYLAMIDE (CROSSLINKING AGENT)**

adversely affect polymerization and subsequent gel properties.

(b) **Initiation of polymerization**

Radicals required to initiate polymerization may be produced by several methods. The most common system uses a chemical source of radicals. Ammonium persulphate produces free oxygen radicals in solution by a base catalysed process, using N,N,N',N'-tetramethylethylenediamine (TEMED). Free radicals can be generated photochemically by use of riboflavin which decomposes to leucoflavin (also by base catalysis). Since free bases such as TEMED are required, polymerization may be delayed or prevented at low pH's; alternative catalyst systems for polymerization at low pH have been employed [146].

The rate of polymerization and resulting gel properties depend upon concentrations of initiators employed. Generally, the lowest catalyst concentrations which produce visible gelation within 15-20 minutes using TEMED and 30-60 minutes using riboflavin-based systems have been found acceptable [147]. Excess amounts of initiators have been shown to damage proteins and cause artefacts in PAGE [148,149].

(c) **Polymerization temperature**

The polymerization reaction is exothermic to the extent that highly concentrated or large diameter polyacrylamide gels produce considerable heat. The high temperatures lead to cavities being formed in the gel as a result of dissolved gases; this effect has been shown to diminish when the temperature of the gel solution is reduced to 6$^{o}$C prior to polymerization [150]. However,

polymerization at lower temperatures (4°C) may result in the production of turbid, increasingly porous and inelastic gels [147], these effects possibly being due to increased hydrogen bonding of the monomer. However, Gressel et al. [151] found that electrophoretic mobilities in PAGE at 22°C increased with increasing polymerization temperature.

(d) **Oxygen**

Molecular oxygen inhibits the formation of polyacrylamide gel by acting as a free radical trap; degassing of monomer solutions is therefore essential [147]. However, the conversion of riboflavin from the flavo to the leuco form actually requires a small amount of oxygen [152] although excessive oxygen will inhibit even such photochemically initiated polymerization.

(e) **Monomer concentrations**

The concentrations of monomers employed will influence polymerization and the properties of the resulting gels. Hjerten [153] introduced the numerals T and C to define gel monomer concentrations where T denotes the total percentage concentration (w/v) of both monomers (acrylamide and Bis) and C is the percentage concentration (w/w) of crosslinker relative to the total monomer concentration, T. It is generally accepted that the practical range for monomer concentration is between 3T and 30T [147].

### 1.5.4.1.1. Polyacrylamide gel density (porosity)

In contrast to electrophoresis in liquid media, polyacrylamide gel is capable of distinguishing molecular species on the basis of

size as well as charge, this phenomenon is termed "the molecular sieving effect". Polyacrylamide gel porosity is variable over a wide range, indeed gel concentration (T) is selected according to weight of molecules undergoing electrophoresis, macromolecules of over 100,000 Daltons being separated by 3-5T gels (table 1.1.) and polypeptides of molecular weight as low as 2000 experiencing molecular sieving in 30T polyacrylamide gels [154]. A linear relationship is generally found [155,156] in electrophoresis of proteins, between the logarithm of relative mobility and polyacrylamide gel concentration (T).

**1.5.4.1.2. Pore theory**

Smithies [157] and Ornstein [158] used the terms 'pore' and 'pore size' in attempting to explain the retardation of a particle migrating through a gel network; a number of theoretical approaches have been employed in calculation of average pore sizes:

Ornstein [158] calculated that 7.5T and 30T polyacrylamide gels should have mean pore diameters of 5 nm and 2 nm respectively. Raymond and Nakamichi proposed equation 1.18, where the average pore diameter, $\bar{p}$, is inversely proportional to the square root of gel concentration (T) of the polymer with molecular diameter d[159]

$$\bar{p} = \frac{kd}{\sqrt{T}} \qquad \text{(equation 1.18)}$$

where the concentration of the gel is defined by equation 1.19

$$T = \frac{k^2 d^2}{\bar{p}^2} \qquad \text{(equation 1.19)}$$

where k is dependent upon the geometric configuration of the gel and has a value of 1.5 if crosslinking of polymer strands at right angles is assumed. Thus in a 5T polyacrylamide gel (with chain strands of 0.5 nm diameter) a pore diameter of 3.8 nm is calculated.

Tombs [160] related pore size, gel concentration and diameter of cylindrical strands by equation 1.20; for example, a pore size of 2.9 nm is obtained for a 5T polyacrylamide gel of 0.5 nm cylindrical strand diameter.

$$\bar{p} + d = \frac{\sqrt{3\pi}5d}{\sqrt{T}} \qquad \text{(equation 1.20)}$$

Considering that many calculated mean pore sizes cannot account for the passage of large protein molecules, Tombs [161] introduced the concept of a "limiting pore size" which is dependent on the proportion of pores larger than a limiting size which the protein molecules cannot quite enter.

A number of workers do not favour the pore concept, for example Hjerten [162] prefers the term "size sensitivity" whilst Hedrick and Smith [156] consider that "sieving" does not describe the complex physical process responsible for the phenomenon.

Pore theories assume polyacrylamide gel to be composed of a rigid matrix not displaced by a migrating particle; however Gordon [152] considers that macromolecules may "bend" gel fibres during electrophoresis since proteins show very slow rates of diffusion in the same gels.

#### 1.5.4.1.3 Electrophoretically migrating species size and shape

Electrophoretic mobility is usually found to decrease with increasing particle size. For example, Morris and Morris [163] demonstrated such a relationship between mobility and mean molecular radius (1.57 to 3.61 nm) of 8 proteins during PAGE. Macromolecular conformation is also important, most proteins are considered to be prolate ellipsoids [164]. An extreme case is DNA, a long and rigid molecule whose electrophoretic mobility is little affected by changes in gel porosity since it is believed to migrate in a "serpentine" fashion [165] through gels, restricting the passage of spherical molecules of higher molecular weight.

Although electrophoresis is generally used in the separation of polypeptides, smaller molecules such as antibiotics have been separated and identified by means of gel electrophoresis [56,166].

#### 1.5.4.1.4. Crosslinking in polyacrylamide gels

The concentration of crosslinker (C) influences electrophoretic mobility in polyacrylamide gels. For example, Rodbard et al. [167] found that protein (e.g. BSA) mobility in PAGE fell with rising Bis concentration up to 5-8C and then increased as Bis was raised from 10 to 50C.

With higher proportions of crosslinker, the polymer chains are believed to become crosslinked into increasingly large bundles with large spaces between them so the effective pore size is increased [154,168]. Previously, it had been stated by Raymond and Nakamichi [159] that Bis provided only mechanical stability to the gel and did not influence pore size.

Although Bis is most widely used, alternative crosslinkers are

available e.g. N,N'-diallyltartdiamide (DATD) which was found to improve mechanical stability and adherence to glass of polyacrylamide gels compared with Bis [169].

### 1.5.4.1.5. Electron microscopy of polyacrylamide gels

Scanning electron microscope studies of freeze-dried polyacrylamide gels [170,171] suggest a heterogeneous structure consisting of 2 to 10 um thickness walls surrounding pores of similar dimensions, with wall thickness increasing as T is raised. This and further work [172,173] suggests that any "sieving" action must occur within a finer infrastructure in the walls which are possibly an artefact of electron microscopy preparation techniques in any case.

### 1.5.4.2. Swelling of polyacrylamide gel

Polyacrylamide gels usually swell in water or aqueous buffer solution and this phenomenon might be expected to considerably influence molecular sieving behaviour. However, polyacrylamide gels for electrophoresis have been little studied in terms of their swelling behaviour.

#### (a) Mechanism of swelling

A solvent's chemical potential is lowered on addition of solute, but application of pressure to the solution can restore the solvent's original chemical potential. The crosslinked macromolecular network of polyacrylamide gel can be considered as a solute which imbibes water (solvent). The resultant swelling is opposed by tension in the

network and water imbibition ceases when the swelling pressure is equal to the osmotic pressure of the gel [7].

(b) **Some factors affecting swelling of polyacrylamide gels**

Boyde [174] confirmed the earlier findings of Richards and Lecanidou [175] that a minimum of polyacrylamide gel swelling occurs at 9-15 T and also found that high initiator concentrations during polymerization and high polymerization temperatures favoured swelling. Richards and Temple [176] observed that swelling decreased with increased crosslinking, C, and proposed a theory indicating a relationship between swelling and polyacrylamide gel turbidity. However, polyacrylamide gel swelling inconsistent with this theory has been reported [178].

Changes in the swelling of polyacrylamide gels 'cured' (left in polymerization mould) for different lengths of time are believed to be due to the formation of a heterogeneous gel structure [168,177]. No discussion regarding swelling of polyacrylamide gels during electrophoresis has been published.

### 1.5.4.3. Electrophoretic techniques

PAGE finds greatest application in the separation of mixtures of biological macromolecules. These are usually applied as distinct bands which gradually separate during the course of electrophoresis into their components. From this simple basis, numerous specialised PAGE procedures have been developed; some of the more commonly used techniques are discussed below.

(a) **Molecular weight determination**

A plot of gel density (T) versus the logarithm of a particle's electrophoretic mobility (U) or relative mobility yields a straight line, where the slope of the line is termed the retardation coefficient, $K_R$. This relationship was first demonstrated in starch gels by Ferguson [178] and later applied to polyacrylamide gels by Hedrick and Smith [156] who found a linear relationship between $K_R$ and molecular weight at least over the range 45,000-500,000 Daltons. Thus by reference to a number of standard proteins, the molecular weight of an unknown protein may be determined by measurement of its mobility (or relative mobility).

(b) **Gradient electrophoresis**

Polyacrylamide gels with a gradient of increasing acrylamide concentration, T, are used for molecular weight determinations. During electrophoresis, proteins migrate until the decreasing pore size impedes further progress, thus "bands" of different molecular weight fractions result.

In isoelectric focusing [179], a pH gradient is established along the gel and proteins migrate until they align themselves at their isoelectric point (pI).

(c) **Preparative electrophoresis**

Preparative electrophoresis involves the scale up from microgram quantities of sample (as applied in analytical electrophoresis) to milligrams of protein mixtures where recovery is necessary. Preparative techniques may be divided into 2 main classes according to recovery procedure:

(i) Extraction of gel fractions: after electrophoresis, separated components are localised by staining of a reference gel. After cutting the gel into appropriate fractions, the sample is eluted by diffusion or further electrophoresis.

(ii) Continuous electrophoretic separation and elution: separated components migrate to the end of the gel where they are entrained by a continuous flow of buffer and are collected individually.

### 1.5.4.4. Materials and methods in PAGE

#### (i) Electrophoresis apparatus and gels

Although designs of equipment for PAGE are varied, they are based on 3 geometric configurations of the gel: (a) horizontal gel slabs, (b) vertical gel rods or cylinders, (c) and vertical gel slabs.

Gel composition is determined by intended application; for example, Andrews [180] suggests polyacrylamide gel concentrations suitable for separation of macromolecules according to macromolecular weight (table 1.1).

| Gel concentration (T) | Optimum molecular weight range |
|---|---|
| 3-5 | >100,000 |
| 5-12 | 20,000-150,000 |
| 10-15 | 10,000-80,000 |
| 15+ | <15,000 |

**Table 1.1 Separation ranges of polyacrylamide gels of various gel concentrations, T.**

Electrophoretic apparatus usually comprises 2 electrolyte reservoirs which are connected by a gel, rod gel electrophoresis is discussed in greater detail in section 5.

**(ii) Localisation of separated components after electrophoresis**

A large number of staining mixtures including both universal and specific stains are available [181]. Densitometry is usually employed for quantitative determination of separated components, where either the gels themselves, or negatives of gel photographs, are scanned. Components are often localised by radioactive labelling and fluorescent 'markers' have been used to monitor protein migration during electrophoresis (Appendix 1). Migration velocities are not equal but proportional to electrophoretic mobility, thus most workers express PAGE results as "relative mobilities".

**(iii) Buffers for electrophoresis**

PAGE may employ "continuous" or "discontinuous" buffer systems. In the former, the same buffer ions are present in the gel as in the electrolyte reservoirs. In contrast, discontinuous (or multiphasic) buffer systems use different buffer ions within the gel compared to those in the electrolyte reservoirs. Most multiphasic systems have discontinuities in both buffer composition and pH. Ornstein [158] and Davis [182] introduced the concept of multiphasic buffering to improve resolution of sample components by concentration of proteins into extremely narrow zones.

Specially formulated synthetic mixtures of "carrier ampholytes" are utilised as buffers in isoelectric focusing (section 1.5.4.3.b.) to maintain a stable pH gradient along the gel [179].

The net charge on a protein is determined by the pH of its environment, thus buffer pH will influence electrophoretic mobility and is selected to give optimal separation of protein mixtures. A universal buffer for electrophoresis is not available and a very large number of different acids and bases can be used.

Buffer ionic strength will also influence electrophoretic mobility since it affects the electrokinetic potential (section 1.5.2.). Ionic strengths in the range 0.025 to 1M are usually employed in PAGE. Low ionic strengths are found to permit high migration rates whilst higher ionic strengths give slower rates of migration but sharper zones of separation [135]; Gaal et al. [136] consider that observations inconsistent with these findings are due to the influence of ionic strengtn upon factors such as molecular shape and dissociation.

The higher the ionic strength of a buffer, the greater is its conductivity, thus more heat is generated. Temperature increases result in a lowering of gel viscosity and greater sample diffusion. Control of temperature during electrophoresis runs is therefore vital.

(iv) **Power supplies for PAGE**

A direct current (DC) power source is required to create an appropriate electric field for electrophoretic migration to occur. The resistance of the electrophoresis cell may change during the course of an electrophoresis experiment due to factors such as ionic migration, electrolysis and ohmic heating effects. If the electrical resistance changes, then either the voltage or the current

or both must also change. Most workers agree that either a constant voltage or constant current power source should be used for PAGE [154,183,184], the latter being preferred. However voltage and current will alter as cell resistance changes with both constant current and constant voltage supplies respectively; recently, constant power supplies and 'pulsing' sources have become available [183,184].

Generally, voltages of at least 100 V or currents of 1 to 3 mA per gel are used in PAGE separations; power supplies for electrophoresis rated at thousands of volts and hundreds of milliamps are available [184].

Application of 40 V to slab gels and less than 20 V $cm^{-1}$ in paper electrophoresis have been termed "low voltage" techniques [180,185]. Rod PAGE using voltage levels employed in the present study (less than 10 volts) has not previously been reported.

Higher voltages are usually preferred since the increased field strength produces higher migration velocities and thus shorter running times (equation 1.12) as well as limiting band broadening caused by diffusion. However,the detrimental effects of extra heat generated by the increased current impose practical upper limits to the applied electric field.

Since the absolute electric field, E, is not easily determined in PAGE [196] and the technique is generally comparative in nature, many workers use applied voltage or current to describe electrophoresis conditions.

### (v) Electrodes and electrolysis

Electrochemical reactions occur at the electrodes during electrophoresis. The anode naturally becomes acidic, and the

cathode naturally becomes alkaline under the influence of an electric current [179]. The anode and cathode reactions are as follows.

Anode reaction

$$6H_2O \longrightarrow O_2 + 4H_3O^+ + 4e^-$$

Cathode reaction

$$4H_2O + 4e^- \longrightarrow 2H_2 + 4OH^-$$

With multicomponent buffers as electrolytes, electrode reactions may be complex. Platinum is usually employed as electrode material in PAGE.

In micro-electrophoresis [186], platinum black electrodes are used to minimise gas evolution by adsorption.

Electrode polarization may occur during electrophoresis. The total polarization resulting from 2 factors; chemical and concentration polarization. In concentration polarization, changes in local electrolyte concentrations may alter electrode potentials. The chemical reactions involved in the discharge or production of an ion give rise to chemical polarization.

The change in electrode potential resulting from polarization is called "overvoltage", or "overpotential".

Polarization is not usually considered in PAGE, but can be reduced by stirring, decreasing the current density and decreasing electrolyte concentration [187].

# 2. MATERIALS AND ANALYTICAL METHODS

## 2.1. Materials

### 2.1.1. Drugs

**Crystalline Porcine Insulin,** specially purified for research $2.85 \times 10^4$ International Units $g^{-1}$ (lot numbers 615-07J-256-A and 615-3UO-130) was obtained as a gift from Lilly Research Centre Limited, Windlesham, U.K.

**Iodine-125 labelled Insulin,** batch number B282 (Amersham International plc, Amersham, U.K).

**Bovine Serum Albumin (BSA),** batch no. 83C-0340 (Sigma Chemical Co. Ltd., Poole, U.K.).

**Tritiated hyoscine methyl chloride** (Amersham International plc), batch TRK.666/8.

**Hyoscine methyl bromide,** batches 43F-0501 and 123F-0632 (Sigma Chemical Co. Ltd.) was converted into the chloride salt before use by means of an ion-exchange technique: An ion-exchange resin (Amberlite IRA 400 Cl, BDH Chemicals, Poole, U.K.) was packed as a slurry (in distilled water) into a 15 cm length, 1 cm internal diameter glass column. Plugs of glass wool were placed at each end of the packing material. The resin was activated by allowing 50 $cm^3$ of concentrated hydrochloric acid (BDH Chemicals) to slowly run through the column followed by 500 $cm^3$ of distilled water to remove excess acid. 50 $cm^3$ of a solution of hyoscine methyl bromide (approximately 0.25 mol $dm^{-3}$ was run through the column 3 times and evaporated to dryness at 50°C. On the basis of a chemical test for chloride [189] the converted salt was used without further purification.

**Propranolol hydrochloride B.P.** was received as a gift from A.H. Cox and Co. Ltd., Barnstaple, N. Devon, U.K.).

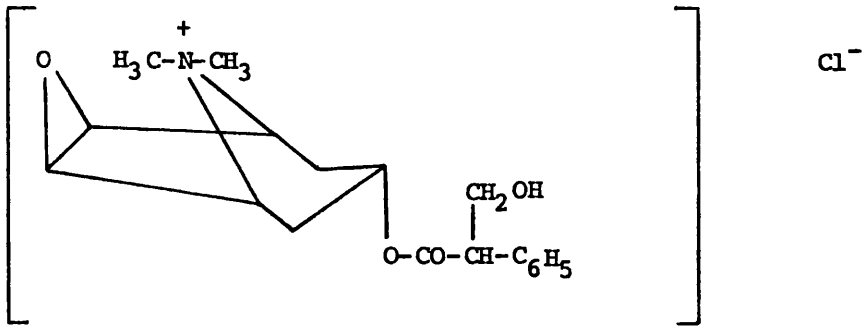
**Hyoscine hydrobromide,** batch 72F-0567 (Sigma Chemical Co. Ltd.).

Some of the physical and chemical properties of the above drugs which may be of importance in relation to electrophoretic release characteristics are given below:

(i) **Bovine serum albumin (BSA):** a protein often employed as a reference standard in PAGE. It has a molecular weight of approximately 57000, an isoelectric point (pI) of 5.1 at 25°C [181] and is prolate ellipsoid in shape with approximate dimensions 15 x 3 nm [190].

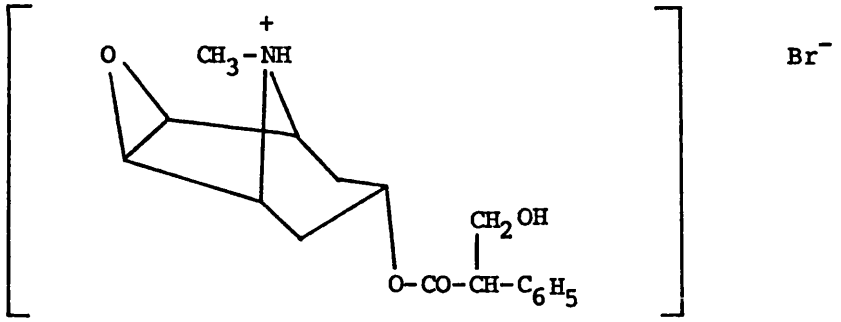
(ii) **Porcine insulin:** a hormone composed of 2 (A and B) polypeptide chains of 5777 molecular weight [191]. The insulin dimer is the prevalent form in solution [192] with approximate dimensions of 2 x 4 nm and an isoelectric point of 5.7 [181]. Porcine insulin is produced commercially from the pancreas of the pig and used in the treatment of diabetes in man.

(iii) **Hyoscine methyl chloride** (m.w. 354) is a quaternary ammonium compound and thus incompletely absorbed from the gastro-intestinal



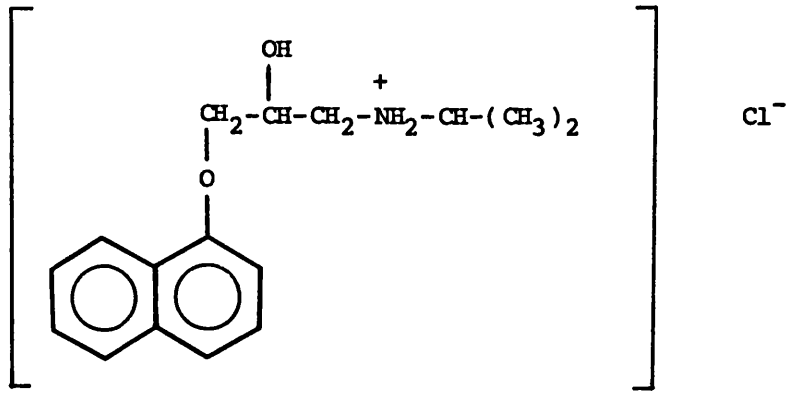

tract; the bromide salt has been used in the treatment of gastric and duodenal ulcers [193].

(iv) **Hyoscine hydrobromide** is a tertiary ammonium tropane



alkaloid of m.w. 384.3 with an acid dissociation constant (pka) of 7.35 at 30°C [39]. It has central and peripheral anticholinergic actions and has been used for pre-operative medication. Hyoscine hydrobromide is found to permeate through skin [39] and has been used for the prevention of motion sickness by oral and transdermal administration (section 1.2.4.4).

(v) **Propranolol hydrochloride** has a molecular weight of 295.8



and pKa of 9.5 [194]. Propranolol has a specific blocking action on the adrenergic beta receptors and inhibits sympathetic stimulation of the heart; it is used in the treatment of cardiac arrythmias, angina pectoris and arterial hypertension [193]. Usually administered orally, it has recently been suggested that transdermal delivery of propranolol may be possible [67].

### 2.1.2. Reagents used in preparation of hydrogels

The following monomers were used as received:

**Acrylamide** (batch no. A/0977/30), specially purified for electrophoresis (Fisons plc, Loughborough, U.K.).

**N,N'-methylene-bis-acrylamide** (Bis), electrophoresis grade (batch M/4811/01, Fisons plc).

**2-Hydroxyethyl methacrylate (HEMA)**, batch no. 22807 1112 (Fluka A.G., Fluorochem Ltd., Glossop, U.K.).

**Ethylene glycol dimethacrylate** (EGDMA), batch no. 201529 381 (Fluka A.G.).

Chemical catalysts were utilised without further purification:

**Ammonium persulphate A.R.** grade, batch 9062620C (BDH Chemicals).

**N,N,N',N'-tetramethylethylenediamine** (TEMED), S.L.R. grade, batch 5864190B (BDH Chemicals).

### 2.1.3 Materials used in high performance liquid chromatography (HPLC)

1-heptanesulphonic acid sodium salt, batch 23 (HPLC grade, Fisons plc).

Tetra-n-butylammonium hydroxide (40%) solution, batch 981190F (Fisons plc).

Methanol (HPLC grade), Fisons plc.

### 2.1.4. Skin

Skin samples were obtained from Caucasian cadavers (aged 60 years or more) and were excised from the abdominal region. Samples were stored in sealed polythene bags at -4 to -5$^{o}$C and used within one month (see section 7.1.1.).

### 2.1.5 Miscellaneous materials

4-Phenylspiro (furan-2-[3H], 1'-phthalan)-3,3'-dione or fluorescamine (Sigma Chemical Co. Ltd.).

Scintillation liquid:'Optiphase safe', batch no. 06 (for LKB by Fisons plc, Loughborough, U.K.).

All other chemicals, solvents and reagents were of "analytical reagent" quality wherever possible.

## 2.2. Analytical methods

### 2.2.1 U.V. spectrophotometric assay of porcine insulin

Most polypeptides exhibit wavelengths of maximum absorbance in the U.V. region and therefore ultraviolet spectrophotometry is often utilised as a convenient method for the assay of proteins.

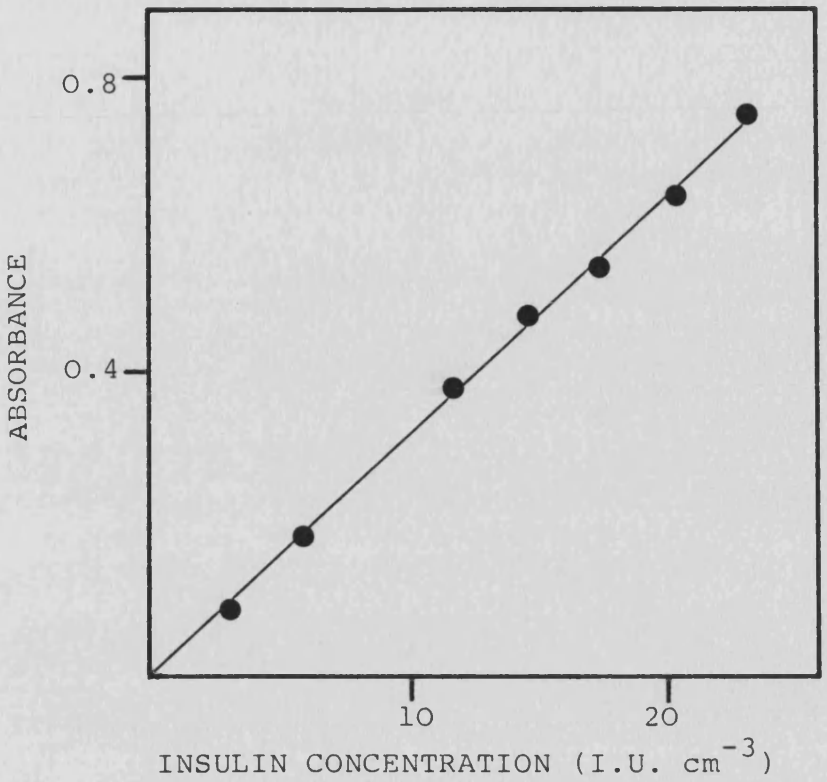
Drug solutions were prepared by dissolving insulin in 0.025M phosphate buffer (pH 7.4) to provide a concentration range of 2.85 to 22.80 International Units (I.U.) $cm^{-3}$. Using 1 cm path length quartz cuvettes, the absorbance of each solution was determined using a U.V./visible double beam spectrophotometer (model 550S, Perkin Elmer Ltd., Slough, Berkshire, U.K.).

273.5 nm was utilised as the wavelength of maximum absorbance. A calibration curve was constructed (figure 2.1), using the absorbance values obtained for the range of concentrations studied, and this indicated compliance with the Beer-Lambert Law. Insulin concentrations below 2.85 I.U. $cm^{-3}$ produced very low absorbance readings whilst concentrations greater than 22.80 I.U. $cm^{-3}$ failed to comply with the Beer-Lambert Law. Thus gamma counting (section 2.2.2) was preferred and used for insulin assay during this study unless otherwise stated.

### 2.2.2. Assay of insulin by a gamma counting method

#### (a) Outline of technique

Iodine-125 is a radionuclide which disintegrates with the emission of gamma radiation. In a gamma counter of the solid scintillator type, the gamma rays cause the scintillant (e.g. sodium iodide crystals) to fluoresce and by conversion of these photons into electrical pulses, the counter quantifies the number of




| | |
|---|---|
| SLOPE | 0.0330 |
| INTERCEPT | -0.0009 |
| CORRELATION COEFFICIENT | 0.9993 |

FIGURE 2.1 CALIBRATION CURVE AND RELATED STATISTICAL DATA FOR U.V. SPECTROPHOTOMETRIC ASSAY OF INSULIN

decay events occurring in a given period of time.

(b) **Insulin assay procedure**

The gamma counting technique used for the assay of insulin was modified from a method described by Richens [195]. The activity of Iodine-125 in test solutions was determined in the following manner:

3 samples, each of 1 $cm^3$, were removed from the solution for assay using an automatic replicating pipette (Gilson Pipetman P1000, Anachem Ltd., Luton, U.K.) and placed into polyethylene sample tubes (10 mm x 50 mm, LKB Wallac Oy, Finland). Gamma counting was performed using a solid scintillator gamma counter (1275 Minigamma, LKB) and was allowed to proceed for 600 seconds at the preset Iodine-125 energy window. Samples were then returned to the appropriate solution.

(c) **Background count**

The level of background count was periodically monitored by determining the gamma emissions of three 1 $cm^3$ samples of distilled water over a 600 second counting period. A mean value of $25\pm5$ counts per minute (cpm) was obtained and automatically deducted from subsequent samples by the gamma counter.

(d) **Correcting for radioactive decay**

Iodine-125 has a half life (T1/2) of approximately 60 days. The number of atoms not yet disintegrated (Nt) at any time t is given by equation 2.1 [191].

$$Nt = Noe^{-\lambda t} \qquad \text{(equation 2.1)}$$

where No is the number of atoms of an isolated radionuclide present at time zero.

$\lambda$ is the decay constant, a proportionality factor characteristic of the nuclide concerned.

In equal intervals, the number of radioactive atoms decreases by the same proportion; the time interval during which the number decreases by half is known as the half life:

$$T\ 1/2 = \frac{\ln 2}{\lambda} = \frac{0.693}{\lambda} \qquad \text{(equation 2.2)}$$

Equation 2.1 may be modified to incorporate the relationship between the half life and the decay constant giving equation 2.3.

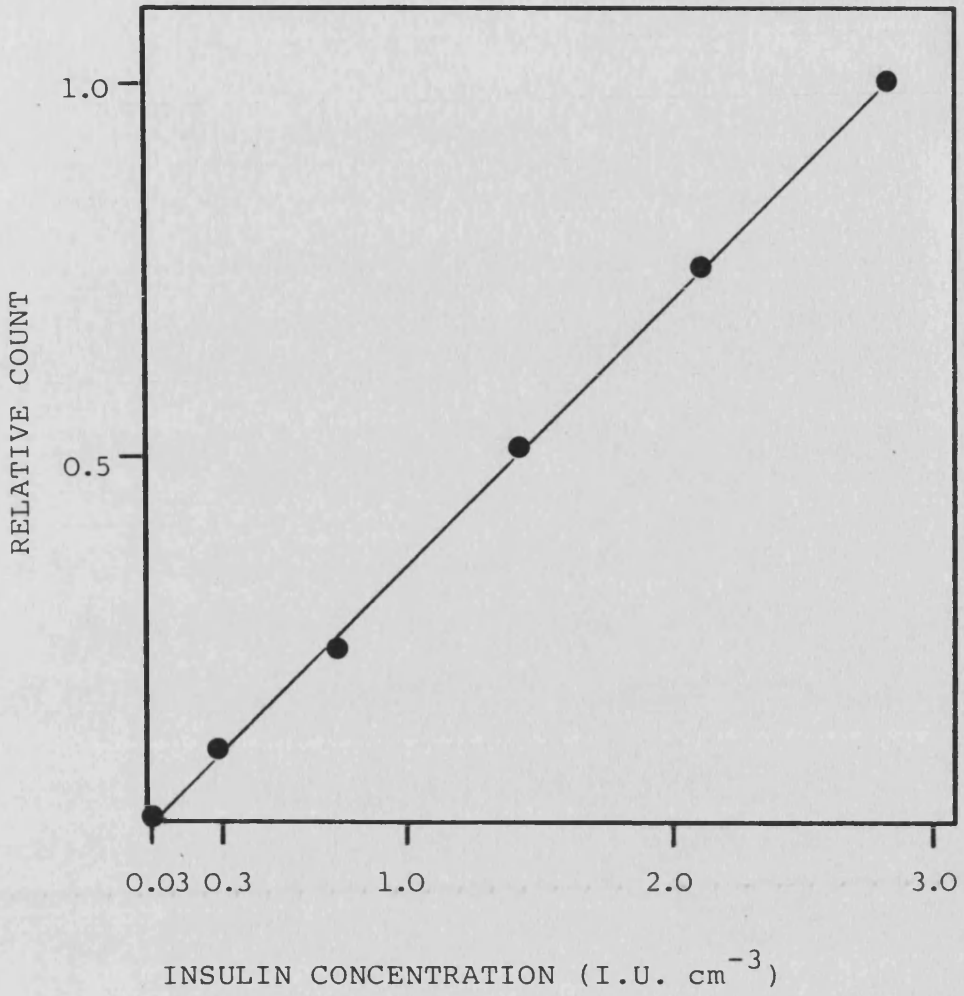
$$Nt = No\ (e^{\frac{-0.693t}{T\ 1/2}}) \qquad \text{(equation 2.3)}$$

Delivery experiments were generally conducted over several days and therefore a decay correction using equation 2.3 was necessary.

(e) **Calibration curve for insulin assay by gamma counting**

To demonstrate a linear relationship between the relative count rate and the relative concentration of insulin in the range to be studied (0.029 to 2.850 I.U. $cm^{-3}$), successive dilutions of a stock solution of Iodine-125 labelled insulin were made. The resulting solutions were assayed and results are shown in the form of a calibration curve in figure 2.2.

Figure 2.2 and related statistical data showed that there was a linear relationship between the count rate obtained from a sample and the concentration of insulin in that sample. Mean counts per minute




| | |
|---|---|
| SLOPE | 0.3568 |
| INTERCEPT | 0.0033 |
| CORRELATION COEFFICIENT | 0.9997 |

FIGURE 2.2. CALIBRATION CURVE AND RELATED STATISTICAL DATA FOR GAMMA COUNTING INSULIN ASSAY

(n = 3) ranged from 105 to 10453 cpm with coefficients of variation not exceeding ±2%.

Subsequently, the activity of a labelled solution of known unlabelled insulin concentration (2.85 I.U. $cm^{-3}$) was determined for each set of experiments and data related to this reference standard.

### 2.2.3. Assay of hyoscine methyl chloride by liquid scintillation counting

#### (a) Basic principles of liquid scintillation counting

Certain radionuclides such as tritium ($^{3}H$) spontaneously disintegrate with the simultaneous emission of a beta particle from the nucleus. This radiation can be measured if the source is mixed directly with a scintillator solution, which usually comprises solvent, scintillators (or fluors) and other additives. The solvent molecules transmit the radiation energy to the scintillators through molecular excitation and the fluors in turn re-emit this energy as photons of a wavelength detectable by a photomultiplier. However, energy transmission between the nuclear particle and the fluor is subject to interference from additives, impurities or indeed the specimen itself; this energy loss is referred to as "quenching". It is necessary to determine the degree of quenching in each sample and correct for it, this is achieved by use of a quench curve.

#### (b) Quench curve construction

A standard of known activity or disintegrations per minute (dpm) is added to the scintillation system and a quenching agent such as

carbon tetrachloride added in increments. After each addition, the sample is counted. From the counts per minutes (cpm) obtained, the counting efficiency (the ratio of cpm to dpm) is calculated after each addition of quenching agent.

By means of an external standard source, the scintillation counter determines the degree of quenching, expresses it as the "external standard ratio" or esr and plots a quench curve (esr versus counting efficiency). A quench curve had been constructed previously for the scintillation system (Optiphase Safe) used in the present study and was stored in the scintillation counter (figure 2.3).

The scintillation counter determines the dpm for an unknown sample by measuring the cpm and the esr (or degree of quenching). Then using the esr and the quench curve, calculates the efficiency and thus dpm is obtained.

(c) **Scintillation counting assay procedure**

The labelling of stock hyoscine methyl chloride solutions was achieved by the incorporation therein of tritiated drug solution of known activity. Tritium activity in samples for assay was determined in the following way:

5 samples, each of 200 $um^3$ were removed from each solution by means of a replicating pipette (Gilson Pipetman P200). The remaining sample solution was restored to volume by the addition of 1 $cm^3$ of appropriate solvent. Each sample was placed into a 5 $cm^3$ capacity polyethylene mini-vial (LKB) and 4 $cm^3$ of scintillant added by means of a bottle pipette dispenser (Oxford Pipettor 400/401). Each vial

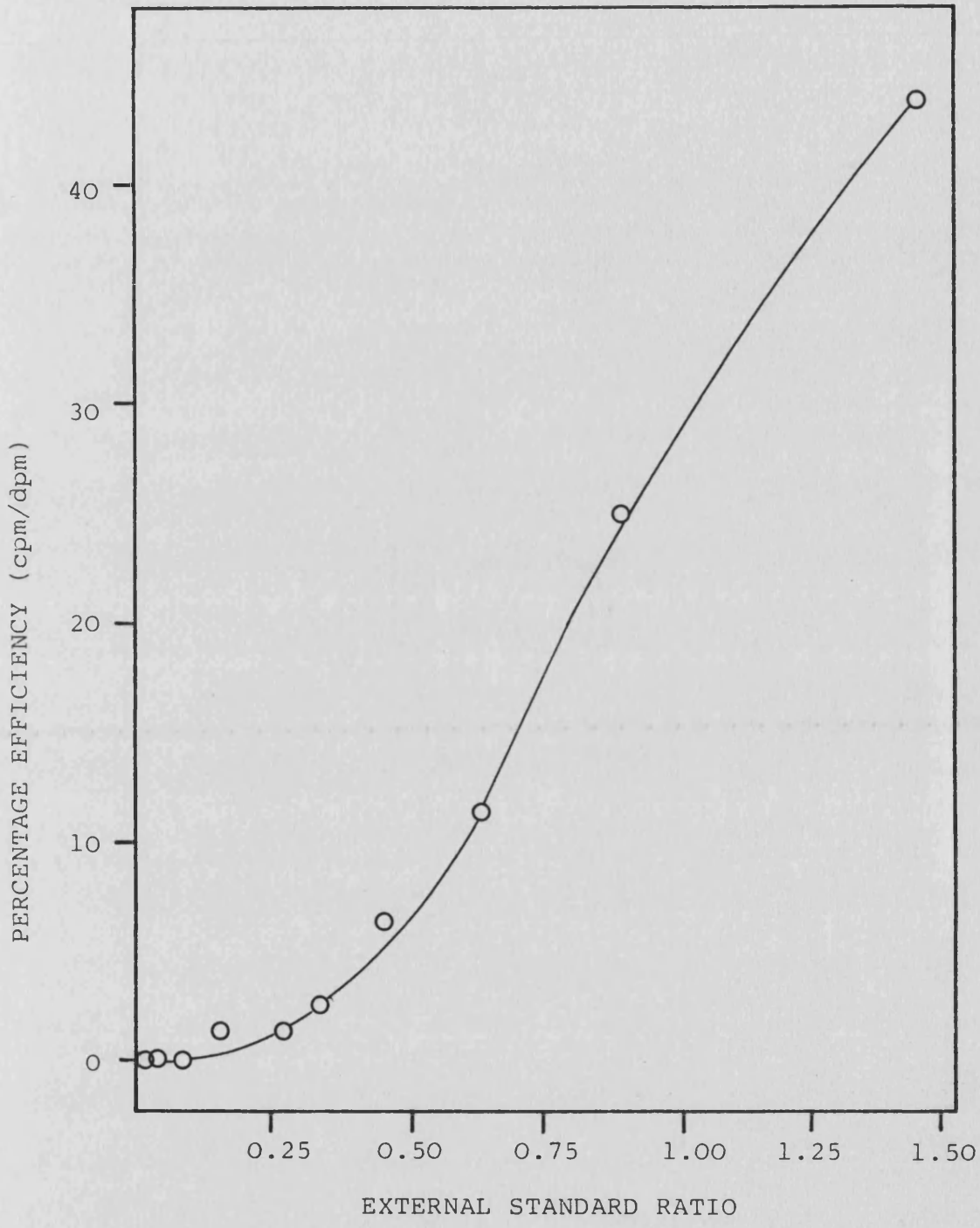



FIGURE 2.3 QUENCH CURVE FOR "OPTIPHASE SAFE" SCINTILLATION SYSTEM

was capped and the mixture shaken for approximately 5 seconds using a "Whirlymixer" (Fisons plc) before being placed into a glass scintillation vial (LKB). Samples were counted for 60 seconds each, with an external standard count time of 30 seconds, in an automated liquid scintillation system (Rackbeta II 1215 LSC, LKB).

The level of background count was determined for each set of experiments by the assay of five 200 $um^3$ samples of distilled water; the mean of the counts obtained was subtracted from sample counts within that group of experiments.

(d) **Reproducibility of liquid scintillation counting technique**

A relatively short count time of 60 seconds (compared with a normal time of 600 seconds or a minimum of 10,000 counts) was employed due to heavy machine usage. The reproducibility of a 60 second counting time was assessed using the following method.

A serial dilution was made (see section (e) below) from a tritiated hyoscine methyl chloride solution of known activity and the resulting solutions counted by the method described above. Mean disintegrations per minute obtained, ranged from 113 to 1210 dpm with coefficients of variation not exceeding $\pm$ 2.1% (n=5). The coefficient of variation in sampling and addition of scintillant were found to be $\pm$0.65% and $\pm$0.15% respectively (Appendix 2). The reproducibility of the liquid scintillation counting technique was considered to be satisfactory for the assay of samples with tritium activity in the range investigated using a count time of 60 seconds.

(e) **Construction of calibration curve for hyoscine methyl chloride assay**

A solution of known hyoscine methyl chloride concentration ($2.82 \times 10^{-4}$ mol $dm^{-3}$) was labelled with tritiated drug; successive dilutions of this mixture were made and the resulting solutions assayed by liquid scintillation counting.

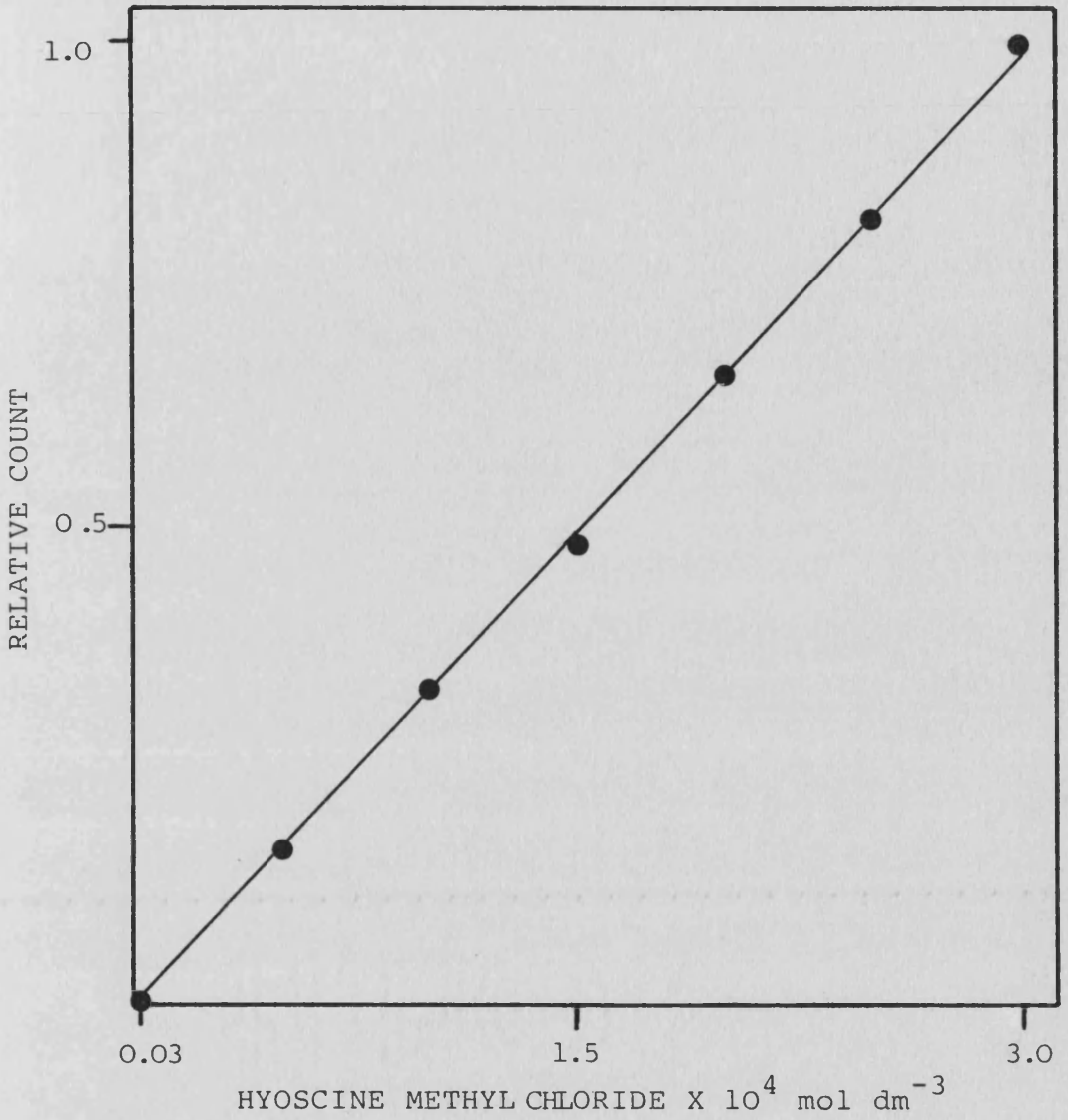
The calibration curve (figure 2.4) and relevant statistical data for the plot show that there is a linear relationship between tritium activity and concentration of hyoscine methyl chloride (in the range $2.82 \times 10^{-6}$ to $2.82 \times 10^{-4}$ mol $dm^{-3}$). The liquid scintillation counting method was thus considered satisfactory for the assay of hyoscine methyl chloride. Subsequently, a reference standard was determined for each set of experiments by the counting of a tritiated donor hyoscine methyl chloride solution of known concentration (section 6.4.2.).

## 2.2.4. Propranolol hydrochloride assay by high performance liquid chromatography (HPLC)

A HPLC method was developed for the quantitative assay of propranolol HCl:

(a) **HPLC conditions for propranolol assay**

A 15 cm length, 0.5 cm internal diameter (i.d.) stainless steel column (HETP, Macclesfield, U.K.) packed with Hypersil octadecanylsulphate (ODS) 5 um reverse phase packing material (Shandon Southern Products, Runcorn, U.K.) was maintained in a constant temperature water bath at $40 \pm 0.1^{\circ}C$.




| | |
|---|---|
| SLOPE | $2.9500 \times 10^3$ |
| INTERCEPT | 0.0008 |
| CORRELATION COEFFICIENT | 0.9999 |

FIGURE 2.4 CALIBRATION CURVE AND RELATED STATISTICAL DATA FOR LIQUID SCINTILLATION COUNTING ASSAY OF HYOSCINE METHYL CHLORIDE

The mobile phase comprised:

| | |
|---|---|
| Tetrabutylammonium hydroxide (40%) | 23 $cm^3$ |
| Phosphoric acid | 40 $cm^3$ |
| Methanol | 500 $cm^3$ |
| Distilled water to | 1000 $cm^3$ |

The mixture was purged with helium for 15 minutes prior to use, giving a solution with a pH of approximately 2.0. A flow rate of 2 $cm^3$ $min^{-1}$ was maintained by a constant metering pump, (LDC Constametric III G, LDC U.K. Ltd., Staffordshire, U.K.), with detection at 288 nm by a variable wavelength U.V. detector (Spectro-Monitor III, LDC/Milton Roy). A 100 $um^3$ injection volume was delivered by means of a stainless steel loop valve (Rheodyne). A peak representing propranolol HCl was observed after approximately 2 minutes 30 seconds (figure 2.5).

(b) **Determination of calibration curve for propranolol HPLC assay**

Injections were made (in duplicate) from a series of solutions of known concentration in the range 2 x $10^{-5}$ to 2 x $10^{-4}$ mol $dm^{-3}$ of propranolol HCl in 50% (v/v) methanol. The height of each peak was measured and the mean peak height for each pair of injections calculated.

The calibration curve (figure 2.6) and related statistical data indicated a linear relationship between peak height and propranolol hydrochloride concentration; the assay was therefore considered satisfactory.

Variations in HPLC column performance with use are inevitable and may manifest as small changes in the calibration slope. Such

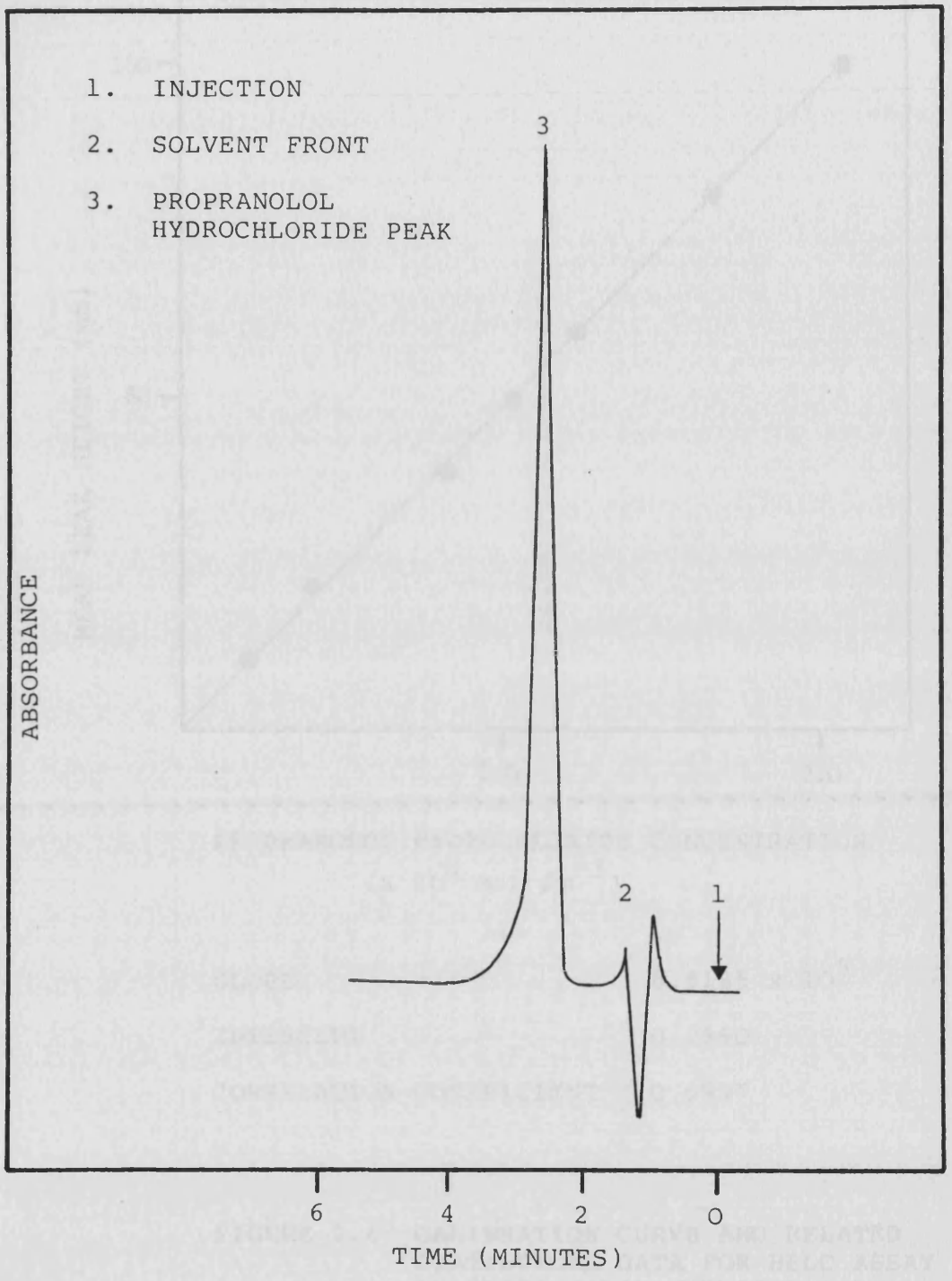
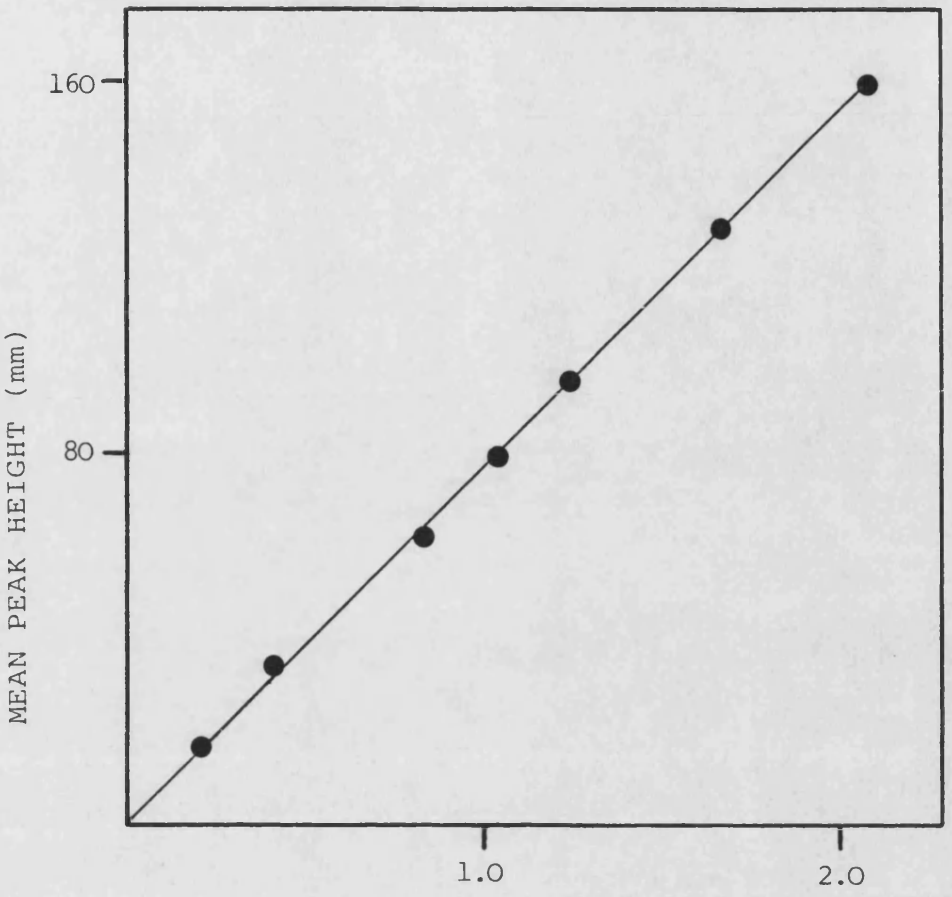



FIGURE 2.5 HPLC CHROMATOGRAM OF PROPRANOLOL HYDROCHLORIDE




| | |
|---|---|
| SLOPE | $8.5185 \times 10^5$ |
| INTERCEPT | 0.2990 |
| CORRELATION COEFFICIENT | 0.9997 |

FIGURE 2.6 CALIBRATION CURVE AND RELATED STATISTICAL DATA FOR HPLC ASSAY OF PROPRANOLOL HYDROCHLORIDE

fluctuations were compensated for by the duplicate injection of a standard solution of propranolol HCl at the beginning of an assay and following every 2 test sample injections (each in duplicate) thereafter. In this way the concentration of drug in sample solution could be calculated by reference to the standard solution.

### 2.2.5. Assay of hyoscine hydrobromide by HPLC

The method employed was based on a technique described by Yamada et al. for the liquid chromatographic assay of belladonna alkaloids [197].

#### (a) HPLC conditions for hyoscine hydrobromide assay

A Hypersil ODS column of 15 cm length and 0.5 cm i.d. (HETP) was maintained in a water bath at $45 \pm 0.1^{o}C$. The mobile phase was composed of 1-heptane sulphonic acid sodium salt (0.005 mol $dm^{-3}$) in 50% (v/v) methanol. This solution was purged with helium for 15 minutes and adjusted to pH 4.0 with glacial acetic acid. A flow rate of 2 $cm^3$ $min^{-1}$ was used (LDC Constametric III G) with detection at 215 nm (Spectro Monitor III, LDC/Milton Roy). Injection was by means of a 50 $um^3$ loop valve and a peak representing hyoscine HBr was observed after approximately 3 minutes 20 seconds (figure 2.7).

#### (b) Calibration curve for hyoscine hydrobromide HPLC assay

A calibration curve was constructed (by a method similar to that described above in section 2.2.4.) for hyoscine hydrobromide in the concentration range $2.6 \times 10^{-6}$ to $2.6 \times 10^{-5}$ mol $dm^{-3}$. From consideration of the curve (figure 2.8) and related statistical data

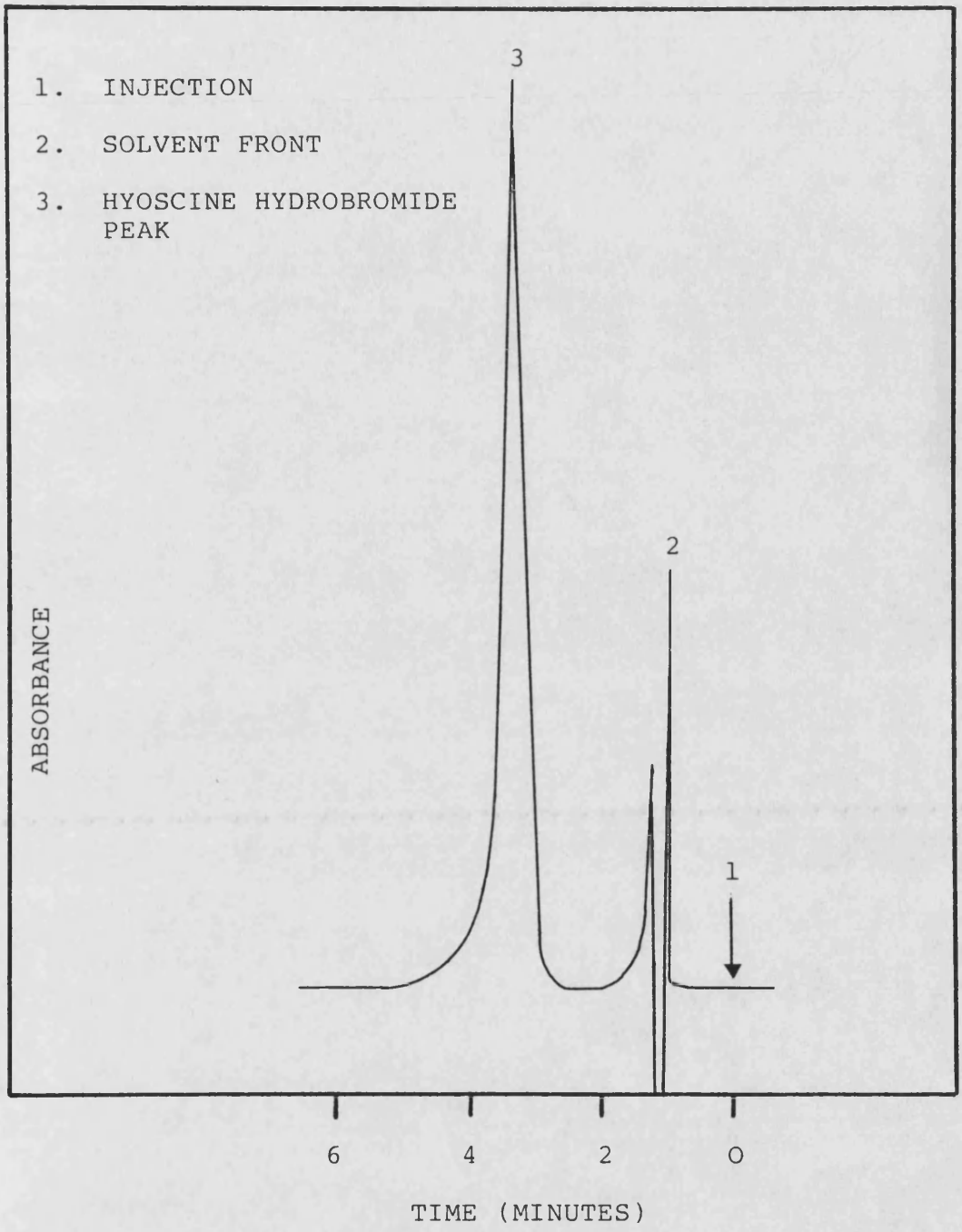
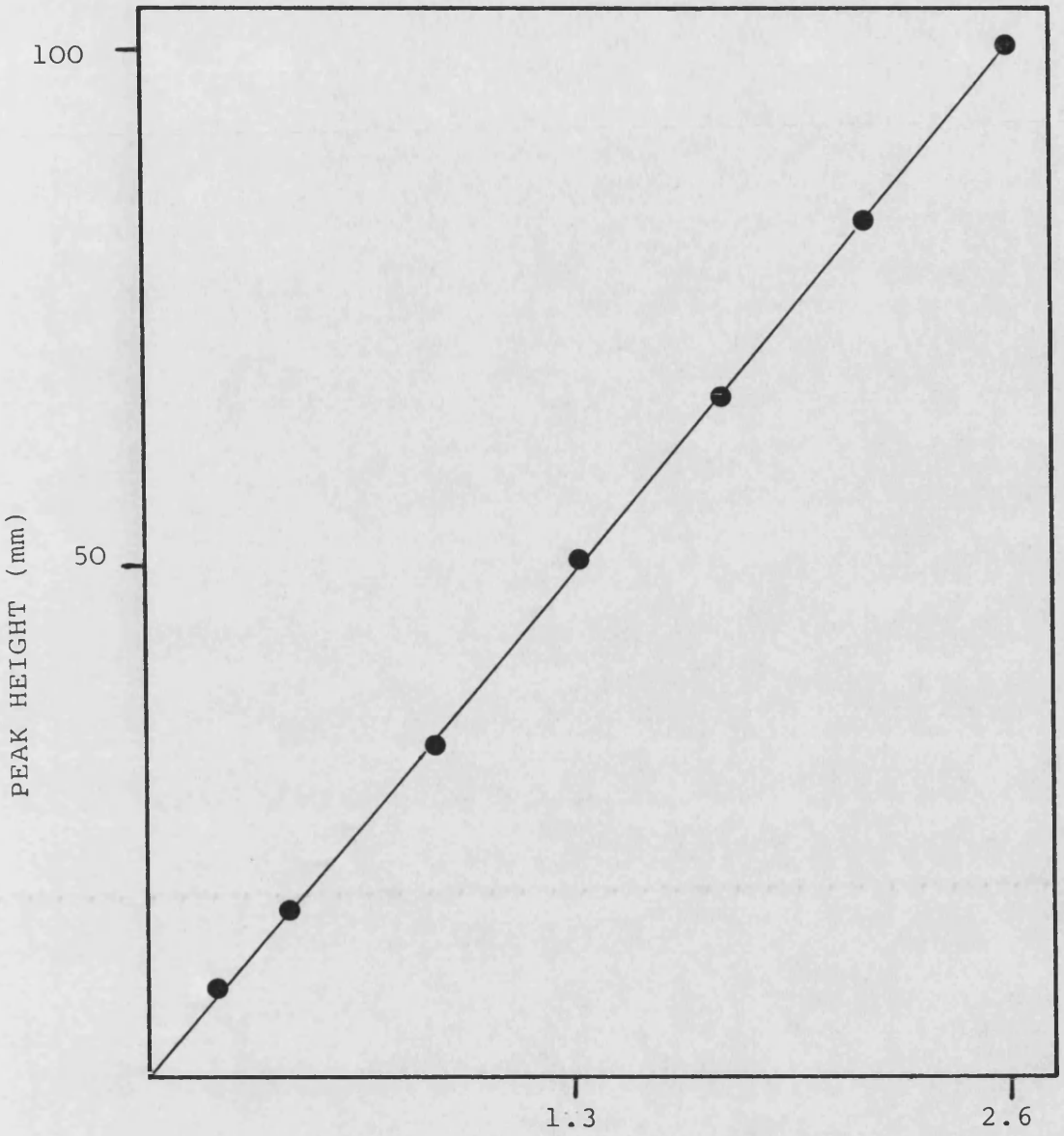



FIGURE 2.7 HPLC CHROMATOGRAM OF HYOSCINE HYDROBROMIDE




| | |
|---|---|
| SLOPE | $3.8313 \times 10^6$ |
| INTERCEPT | 0.3616 |
| CORRELATION COEFFICIENT | 0.9998 |

FIGURE 2.8 CALIBRATION CURVE AND RELATED STATISTICAL DATA FOR HPLC ASSAY OF HYOSCINE HYDROBROMIDE

the calibration was judged to be satisfactory.

### 2.2.6. HPLC of hyoscine methyl chloride

Based on the consideration of the closely related structures of the 2 drugs (section 2.1.1), HPLC conditions employed for the assay of hyoscine hydrobromide (section 2.2.5.) were investigated for the semi-quantitative determination of hyoscine methyl chloride. As shown in figure 2.9, a peak was observed approximately 6 minutes after injection of hyoscine methyl chloride. Increasing the concentration of drug solution injected from $0.7 \times 10^{-5}$ to $1.4 \times 10^{-5}$ mol $dm^{-3}$ resulted in a doubling of peak height. The above HPLC procedure was used to confirm that hyoscine methyl chloride (and not a radiolabelled degradant) was being assayed during electrophoretic release experiments (section 6.4.2.).

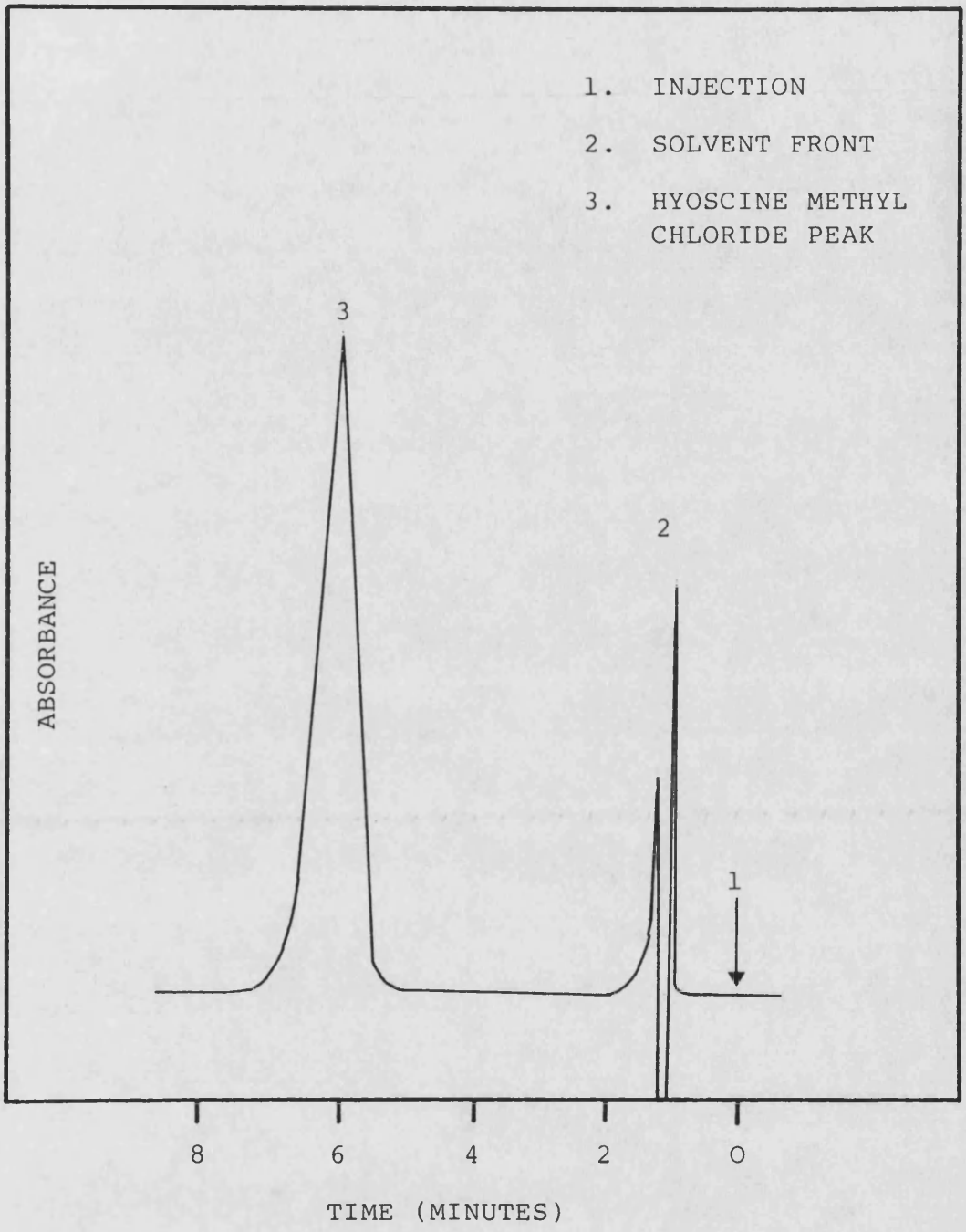



FIGURE 2.9 HPLC CHROMATOGRAM OF HYOSCINE METHYL CHLORIDE

# 3. PREPARATION OF POLYACRYLAMIDE AND POLYHEMA GELS

### 3.1. Polyacrylamide gel preparation

The technique used in the preparation of polyacrylamide gels was based upon those described by various workers [147,150,180] for the preparation of gels for analytical and preparative gel electrophoresis.

#### 3.1.1. Reagents used in polyacrylamide gel manufacture

Monomers, stock solutions of monomers and catalysts used in gel preparation were stored in well-sealed containers, away from light at 4°C. Acrylamide and N,N'-methylene-bis-acrylamide (Bis) solutions were filtered through Whatman No. 4 paper and discarded if unused within one month.

Fresh solutions of ammonium persulphate were prepared daily.

#### 3.1.2. Polyacrylamide gel moulds

Glass tubes were utilised as moulds in the preparation of rod-shaped and disc-shaped gels. These were cleaned by soaking in an approximately 3% w/v solution of sodium dichromate in 90% sulphuric acid for 15 to 30 minutes followed by thorough rinsing in potable and subsequently distilled water. The mould tubes were then oven-dried. One end was sealed with a paraffin wax film (Nescofilm, Nippon, Japan).

Polyacrylamide gel sheets were formed by introducing monomer solution into a mould comprising 2 glass plates separated by a nitrile rubber spacer.

#### 3.1.3. Degassing of monomer solutions

Evacuation of acrylamide/Bis mixtures proceeded for 15 minutes

(using a water vacuum) at a pressure of $4 \times 10^3$ Pa. The solution was then equilibrated at polymerization temperature prior to the addition of reaction initiators.

### 3.1.4. Chemically initiated polymerization of polyacrylamide gels

Ammonium persulphate solution and N,N,N',N'-tetramethylethylenediamine (TEMED) were added, by means of replicating pipettes (Gilson Pipetman P100 and P200) in the following quantities to each 10 $cm^3$ of monomer solution:

| | |
|---|---|
| Ammonium persulphate solution (0.438 mol $dm^{-3}$) | 50 $um^3$ |
| TEMED (undiluted) | 5 $um^3$ |

The mixture was swirled gently for 8 to 10 cycles, i.e. sufficient to mix the initiators completely without the introduction of excessive oxygen. The required quantity of monomer solution was introduced into the mould chambers using replicating pipettes (Gilson Pipetman P1000 and P5000).

The quantities of catalysts, and the degassing time employed ensured that visible gelation occurred after 15 $\pm$ 3 minutes; mixtures failing to gel between 12 and 18 minutes were discarded. Polymerization was allowed to proceed for 30 minutes, gels were then inspected for uniformity of polymerization, and stored in buffer solution of composition to be employed as electrolyte during electrophoresis. Polyacrylamide gels were generally used only after several days of storage (to ensure complete polymerization and osmotic equilibration with buffer solution) and discarded if unused within one month. Buffer solutions were renewed regularly to minimise microbial contamination.

**Typical Formula for Polyacrylamide Gel preparation**

| | |
|---|---|
| Acrylamide | 13.95 g |
| Bis | 1.05 g |
| Ammonium persulphate (0.438 mol $dm^{-3}$) | 250 $um^3$ |
| TEMED | 25 $um^3$ |
| Distilled water | to 50 $cm^3$ |

The formula above produced 30T 7C polyacrylamide gels. The preparation of 3 types of polyacrylamide gels (rods, discs and sheets) is described below:

(a) **Preparation of polyacrylamide gel rods**

Polyacrylamide gel rods were used in rod gel electrophoresis (section 5). In this method, 7.5 cm lengths of glass tubing with an internal diameter of 0.5 cm were utilised as gel moulds. 1.3 $cm^3$ of monomer mixture was placed into each tube (this volume consistently produced gels of 6.5 cm mean length, with a coefficient of variation of less than $\pm$2% (n=8)). A layer 0.2 to 0.3 cm thickness of 10% (v/v) ethanol was carefully layered above the gel solution as quickly as possible using a Pasteur pipette. The ethanol layer was used to reduce surface tension forces at the top of the gel solution so as to produce a flat gel surface and also protect the monomer mixture from atmospheric oxygen which can give rise to an uneven surface (through inhibition of polymerization). Polymerization was allowed to proceed at room temperature; a sharp boundary was observed at the gel/overlay interface when visible gelation had occurred. The gels were flushed with and stored in buffer until use.

(b) **Preparation of polyacrylamide gel discs**

Polyacrylamide gel discs retained within glass moulds were used mainly in electrophoretic release studies described in section 6. To form gel discs, 3 $cm^3$ of gel solution was introduced into glass cylinders of 1.9 cm i.d. and 1.0 cm length. This volume of monomer mixture just overfilled the mould chamber giving a dome shape to the liquid surface. Consequently a flat gel surface resulted without the necessity for overlaying. Polymerization was allowed to proceed in a waterbath at 10°C, higher temperatures resulting in the separation of the gel from the chamber wall (section 1.5.4.1.).

(c) **Preparation of polyacrylamide gel sheets**

Sheets of polyacrylamide gel were formed by pouring approximately 10 $cm^3$ of monomer solution into a mould comprising 2 glass plates (8 cm x 16 cm) separated (by 1.5 mm) by a nitrile rubber spacer. The polymerization was performed in a waterbath at 10°C, the gel sheet was then removed from the mould and rinsed in distilled water before storage. Polyacrylamide gel sheets were used for permeation experiments (section 4.4.2.) and to study the effect of gel thickness upon electrophoretic drug release (section 6.4.2.2.e.).

## 3.2. Chemically initiated polymerization of polyHEMA gels

PolyHEMA gel preparation procedure was based on the chemical catalyst system used in polyacrylamide gel manufacture (section 3.1.4) and a technique reported by Wood et al. [198] for the polymerization of polyHEMA gels.

PolyHEMA gels (10 to 80T) were prepared from 2-hydroxyethyl methacry late (HEMA) monomer and also by co-polymerization with a

crosslinking agent (0.5C), ethylene glycol dimethacrylate (EGDMA) monomer. HEMA and EGDMA were stored at $4^{o}C$ and away from light. Monomer solutions were degassed (3.1.3.) before the addition of 1 $cm^3$ of freshly prepared ammonium persulphate solution (1 mol $dm^{-3}$) producing 50 $cm^3$ of gel mixture. After introducing gel mixture into rod gel moulds (3.1.2.), rubber closures were used to seal moulds and protect the reaction mixture from atmospheric oxygen. Polymerization was allowed to proceed in a $50^{o}C$ water bath for 18 hours. The polyHEMA gels formed were rinsed with and stored in buffer solution.

## 3.3. Polymerization of polyacrylamide and polyHEMA gels by gamma-irradiation

Wood et al. [198] preferred the use of gamma irradiation to initiate polymerization of hydrogels and avoid gel properties associated with residual materials following chemically initiated polymerization.

### 3.3.1 Gamma radiation source dosimetry

Before polymerization was attempted, the dose rate of the gamma source (Caesium - 137) was measured using the Fricke dosimetry system as described by Ahmed [199]. In this technique, gamma radiation causes the conversion of iron (II) ions to iron (III) ions. The oxidation reaction may be quantitatively followed by U.V. spectrophotometric assay, thus a direct measure of energy absorbed by the dosimetry solution due to ionizing radiation is obtained.

Dosimetry solution was introduced into rod gel tubes and these were placed at a height of 40 cm in the gamma source apparatus.

After 30 minutes of exposure to the radionuclide, dosimetry solutions were assayed in $1cm^3$ quartz curvettes at 305 nm using a single beam spectrophotometer (Unicam SP500). A gamma dose rate of 5.5 J $Kg^{-1}$ $min^{-1}$ was obtained.

### 3.3.2. Gamma-irradiation initiated polymerization

Monomer solutions of various formulations were purged with helium for 15 minutes prior to degassing (section 3.1.3) and introduced into rod gel tubes. After sealing with rubber closures, the tubes were subjected to gamma irradiation at ambient temperature for 18 hours.

## 3.4 Results and discussion

### (i) Chemically initiated polymerization

Polyacrylamide gels for PAGE are normally of monomer concentration in the range 3 to 30T (section 1.5.4.1.). In the present study, polyacrylamide gel rods (5 to 30T) and gel discs in the range 10 to 30T were manufactured with relative ease. However, heat generation during polymerization which has been found previously with large diameter gels and those of high monomer concentration [150] occasionally caused some irregularities in 40T polyacrylamide discs. Voids and separation of gel from the mould wall was frequently observed in 50T gel discs, despite the use of low polymerization temperatures ($10^{o}C$).

It was considered that the improved dissipation of heat from polyacrylamide gel sheets during their polymerization was responsible for the high gel monomer concentrations attained (10 to 80T); although polymerized at $10^{o}C$, it was found that gel sheets could also be prepared at room temperature. PolyHEMA gels of 50T and of lower monomer concentration appeared white, whilst 60T, 70T and 80T polyHEMA gels were transparent but often contained numerous voids and showed separation from the mould walls. PolyHEMA gel turbidity arises because the polymerization of a solution containing less than 58% monomer causes the solvation capacity of the resulting polymer to be exceeded, and microsyneresis (phase separation) occurs [7].

### (ii) Gamma-irradiation polymerization

Polyacrylamide and polyHEMA gel rods of an acceptable quality (e.g. uniform appearance) could not be prepared using the methods described in section 3.3. This was believed to be due to a number

of factors: (1) long polymerization times i.e. 18 hours compared with 15 minutes for polyacrylamide gels by chemically initiated polymerization (section 3.1.4). (2) Uneven dose rate along length of gel rod due to gamma source geometry. (3) Lack of temperature control during polymerization. (4) Inadequate maintenance of monomer de-aeration due to long polymerization times.

Chemically initiated polymerization was therefore used to prepare polyacrylamide gels for the remainder of this study.

# 4. CHARACTERIZATION OF POLYACRYLAMIDE GELS

## 4. Characterization of polyacrylamide gels

Some properties of polyacrylamide gels, regarded as likely to influence their functioning as materials employed in electrophoretic drug release studies, were assessed.

### 4.1. Appearance and texture of polyacrylamide gels

Assessment of polyacrylamide gel appearance and texture is discussed in section 4.6.

### 4.2. Scanning electron microscopy of polyacrylamide gels

Despite the wide use of polyacrylamide gels in electrophoresis, little work has been carried out to investigate the structure of the sieving gel. Scanning electron microscopy has found application [170,171], whilst more recently, workers have utilised transmission electron microscopy to view polyacrylamide gels [172].

In the present investigation, polyacrylamide gel structure was examined in a scanning electron microscope (JEOL JSM 35C, Japanese Electron Optics Ltd., Japan).

Gels were cut into strips and rapidly frozen by immersion in liquid nitrogen. Using forceps, frozen gels were removed and fractured by striking with a hammer before being replaced into the liquid nitrogen. Freeze-fractured gels were then freeze-dried at -60°C (Edwards-Pearse Tissue Dryer EPD3, Edwards High Vacuum, Crawley, Sussex, U.K.) for between 8 and 16 hours.

Gel samples were mounted in graphite colloid on 2.6 cm diameter planchettes. A layer of gold was deposited onto the gel surface by means of a sputter coater (Edwards S150B, Edwards High Vacuum). Gels were scanned at an electron beam energy of 20 kV, the gold and

graphite layers ensuring conductance of the sample. Unless viewed immediately after sputtering, samples were stored over dried silica gel in a sealed container to avoid cracking of the gold layer caused by atmospheric humidity changes.

## 4.3. Swelling behaviour of polyacrylamide gels

Polyacrylamide gel discs were prepared as described in section 3.1.4.b. and unless otherwise stated were removed from moulds before use.

Before weighing, gels were rinsed with fresh immersing solution and blotted with tissue to remove superficial liquid. Approximately 30 minutes after visible gelation, gel discs were weighed (initial gel weight) and placed into a beaker containing approximately 50 $cm^3$ of the appropriate soaking solution which was maintained at $30 \pm 0.1^{o}C$ in a waterbath. Polyacrylamide gel discs were removed at regular intervals for weighing, and soaking solutions were changed daily to minimise bacterial infestation and interference from substances leached from the gel (section 4.5.). The degree of swelling was estimated from the observed change in gel weight using equation 4.1.

$$\frac{Wt-Wo}{Wo} \times 100\% \qquad \text{(equation 4.1)}$$

where Wt was the weight of polyacrylamide gel at time t and

Wo was the initial gel weight

Figure 4.1 illustrates the degree of swelling with time of 40T 7C polyacrylamide gels immersed in 0.04M citrate-phosphate buffer (intended for use in electrophoretic release experiments, section 6.4.1.), pH 3.0 and represents a typical gel weight change profile; a quasiequilibrium for swelling was reached after approximately 100 hours. Subsequent data (figures 4.6 to 4.9) show the percentage weight increase of gels after 120 hours, although most determinations were allowed to proceed for a minimum of 200 hours to ensure that equilibrium swelling had been attained. Unless stated to the contrary, each data point (figure 4.1 and figures 4.6 to 4.9 represents the mean of 3 determinations with vertical bars indicating standard deviation about the mean.

### 4.3.1. Factors affecting polyacrylamide gel swelling

#### (a) Ionic strength of immersing solution

30T 7C polyacrylamide gel discs were soaked in distilled water (pH$\simeq$5) and McIlvane's citrate-phosphate buffer of 0.04M, 0.08M, and 0.12M ionic strengths (in the pH range 2.9 to 3.0).

#### (b) Polyacrylamide gel formulation

Polyacrylamide gel discs in the range 10 to 50T (7C) were prepared and immersed either in distilled water, or in 0.04M McIlvane's citrate-phosphate buffer, pH 3.0.

Polyacrylamide gels, crosslinked in the range 2 to 30% (C) with bisacrylamide (Bis) at a constant 30% total monomer (T) were prepared and the swelling after their immersion in 0.04M citrate-phosphate buffer was assessed.

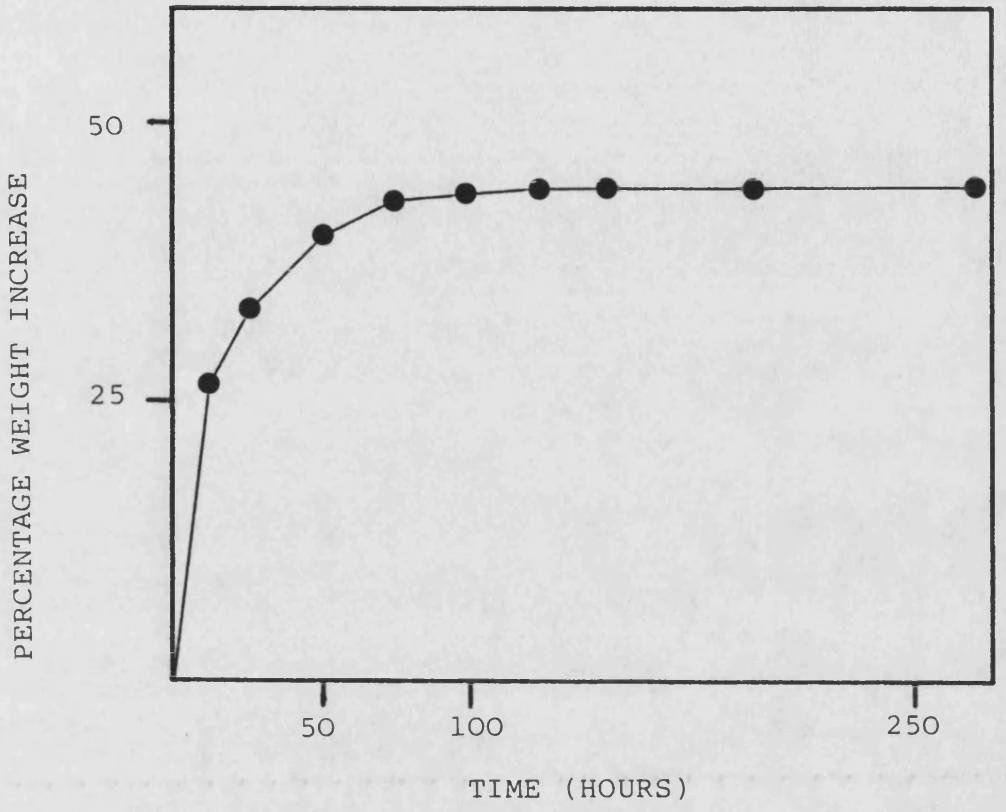



FIGURE 4.1 SWELLING OF 4OT 7C POLYACRYLAMIDE GEL IN 0.04M CITRATE-PHOSPHATE BUFFER (pH 3.0)

(c) **Polyacrylamide gel polymerization temperature**

Polyacrylamide gels (40T 7C) were prepared by allowing chemically initiated polymerization to proceed at the following initial temperatures: 10, 20 and 30°C. The temperature change in the gel mixture during the course of polymerization was not monitored. The swelling behaviour of the polyacrylamide gels formed was investigated by immersion in 0.04 M citrate-phosphate buffer, pH 3.0.

(d) **Polyacrylamide gel swelling during electrophoresis**

Polyacrylamide gel discs (retained within glass moulds) were stored for 3 weeks in either distilled water or 0.04 M citrate-phosphate buffer, pH 3.0; both solutions were renewed regularly. The gel discs were then placed into 2-compartment electrophoretic release apparatus (section 6.1 and figure 6.1) and subjected to pre-electrophoresis at a constant 30 volts (see section 6.2.). 175 $cm^3$ of electrolyte solution (of identical composition to gel storage solutions) was used in each chamber and the apparatus maintained at $30 \pm 0.1$ °C in a waterbath. At timed intervals, the apparatus was dismantled and the gel disc (and glass mould) blotted dry with tissue prior to weighing. After reassembling the apparatus, electrolyte solution was returned to the appropriate compartments and pre-electrophoresis continued. After the last weighing, the polyacrylamide gel was removed from the glass mould and the latter weighed to determine the gel weight.

## 4.4. Drug partitioning and permeation in polyacrylamide gels

The permeability coeffient, P, of a solute in a polymer membrane may be expressed as the product of its diffusion coefficient, D, and its distribution (partition) coefficient, Kd, in the polymer (equation 4.2 [73]).

$$P = DKd \qquad \text{(equation 4.2)}$$

The permeability in hydrogels of solutes of both high and low molecular weights has been widely studied [105-108,200,201]. The techniques employed therein formed the basis of methods utilised in the current investigation.

The distribution coefficients and permeabilities of insulin, hyoscine hydrobromide, propranolol hydrochloride and hyoscine methyl chloride in a number of polyacrylamide gel formulations were determined (section 4.4.1). Polyacrylamide gel sheets prepared by the method described previously in section 3.1.4.c. were used.

### 4.4.1. Determination of drug distribution coefficients (Kd) in polyacrylamide gels

Kd is defined as the ratio of the concentration of solute in the gel phase and solute concentration in the surrounding solution phase, and was determined using a "solution depletion" method described by Lee et al. [201].

Strips of polyacrylamide gel (approximately 0.15 cm x 1 cm x 4 cm) were stored in drug-free buffer solutions for 1 to 2 weeks before Kd determinations. After removal, strips were rinsed with fresh buffer solution and blotted dry with tissue before being placed into 10 $cm^3$ of drug solution in a glass vessel. Drug concentrations

and buffer solutions were as described in electrophoretic release experiments (section 6). The soaking vessels were shielded from light and shaken gently in a waterbath at 30°C for at least 24 hours. The drug content of the solutions surrounding the gels was determined by assays described in section 2.2. The volume of soaked gels was calculated from gel dimensions, the thickness being measured with a micrometer after placing the gel sample between 2 perspex plates of known thickness. The distribution coefficient was calculated using equation 4.3 [201].

$$Kd = \frac{(C_s^o - C_s^s)\, V_s^o}{C_s^s\, Vm} \qquad \text{(equation 4.3)}$$

where $C_s^o$ is the initial drug solution concentration

$C_s^s$ is the concentration of solution surrounding the gel after equilibrium

$V_s^o$ is the initial solution volume

Vm is the volume of the swollen gel.

Each determination was performed in triplicate.

The values obtained for Kd using the above technique were found to be highly variable. Increasing the soaking time in increments, up to one week, and varying the solution to gel volume ratio between 25 and 140 did not improve the reproducibility of the procedure (see section 4.6).

### 4.4.2. Drug permeation in polyacrylamide gels

The diffusive permeation of insulin, hyoscine hydrobromide,

hyoscine methyl chloride and propranolol hydrochloride was determined using an apparatus comprising two chambers of equal volume (as described below in section 6.1. and figure 6.1). A polyacrylamide gel membrane of approximately 1.5 mm thickness was supported by a rubber 'O' ring and held between the 2 compartments (donor and receptor) which were clamped together tightly without causing excessive squeezing of the gel, to provide an exposed membrane area of 4.0 $cm^2$. 175 $cm^3$ of the drug solution of interest (i.e. donor concentrations employed in section 6.) was placed into the donor compartment whilst the same volume of the appropriate drug-free buffer was introduced into the receptor. To reduce boundary layer effects, solutions were stirred at 240 r.p.m. by PTFE paddles; insulin solutions were stirred at a slower speed of 120 r.p.m. to minimise solution frothing. The permeation apparatus was placed into a waterbath maintained at $30\pm0.1^{o}C$ and samples removed from the receptor compartment at timed intervals for assay. Where sample solutions were not returned, the receptor volume was restored by the addition of drug-free solution and the mass of solute removed corrected for when calculating the permeation data.

Each permeation study was performed in duplicate. To illustrate a typical permeation profile, figure 4.2 shows the percentage fraction of hyoscine methyl chloride permeating with time. The lag time is obtained from this type of curve by extropolation of the steady state portion to the time axis (section 1.2.4.3.). From the slope of the straight line region, the permeability coefficient, P, may be calculated using equation 4.4, previously used by Beal to assess the permeability characteristics of polyamide films [97].

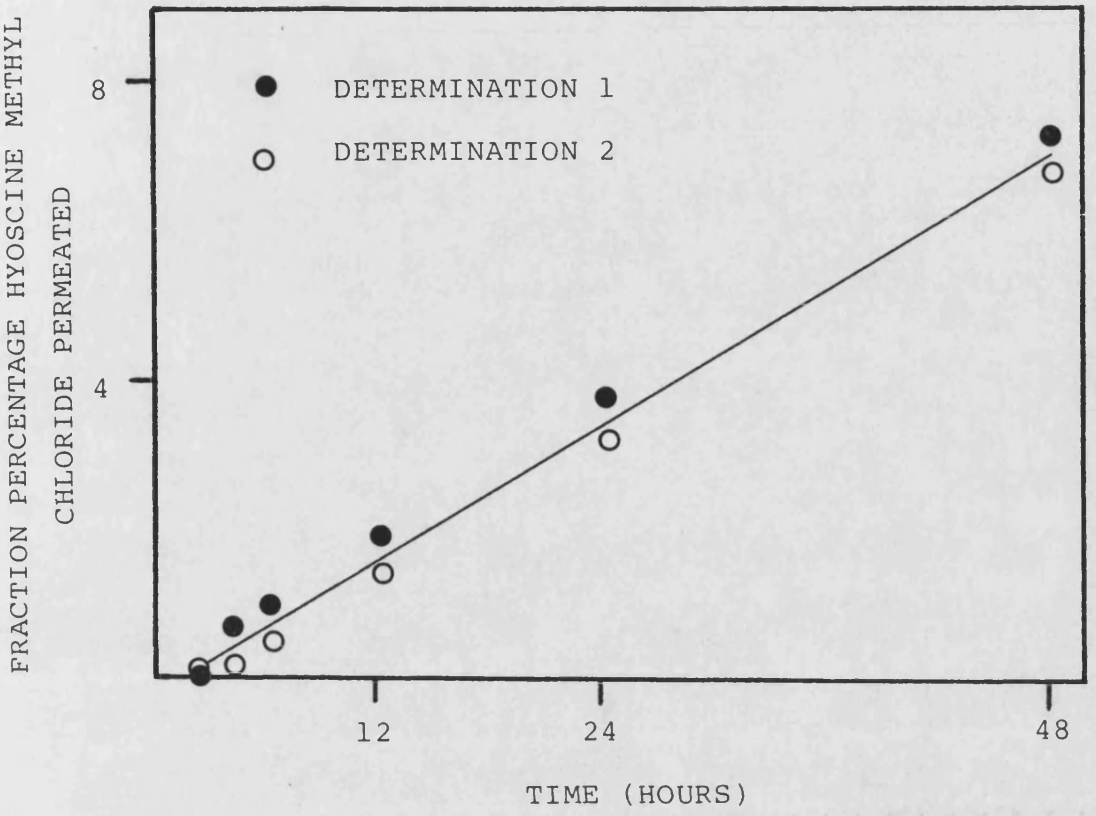



FIGURE 4.2 HYOSCINE METHYL CHLORIDE PERMEATION THROUGH 20T 7C POLYACRYLAMIDE GEL

$$\frac{dC_R^S}{dt} = \frac{P\ A\ C_D^S}{l\ V_R} \qquad \text{(equation 4.4)}$$

where $C_R^S$ is the receptor solution concentration

$C_D^S$ is the donor solution concentration

$V_R$ is the volume of the receptor solution

l is the membrane thickness

P is the permeability coefficient

A is the membrane area

### 4.4.2.1. Effect of gel formulation on the permeability of drugs in polyacrylamide

The effect of increasing gel density in the range 10T to 50T upon the permeability of several drugs through polyacrylamide gels crosslinked to the same extent (7C) was studied. The influence of crosslinking (in the range 4 to 10C) upon permeability of insulin and propranolol hydrochloride was investigated using gels of 30% total monomer concentration (T).

## 4.5. Chemical impurities in polyacrylamide gels

The presence of unreacted catalysts, monomers and other chemicals within polyacrylamide gels may be detrimental to their subsequent performance during electrophoresis. For this reason, purification of gels has been attempted by pre-electrophoresis [202] and by incubation in buffer solution prior to electrophoresis [203], both procedures apparently extracting U.V. absorbing material. In

this study, the U.V. absorbances of solutions in contact with polyacrylamide gels, undergoing incubation were monitored.

### 4.5.1. U.V. absorbance characteristics of reagents used in polyacrylamide gel preparation

The absorbance maxima of solutions of polymerization reagents in citrate-phosphate buffer, pH 3.0 were determined using a double beam spectrophotometer (Perkin Elmer, 550S):

| | |
|---|---|
| Acrylamide | 195 nm |
| Bis | 195 nm |
| Ammonium persulphate | 195 nm |
| TEMED | 205 nm, 254 nm, 259 nm, 265 nm. |

### 4.5.2. Removal of gel impurities by incubation in buffer solution

30 minutes after visible gelation, a 40T 7C polyacrylamide gel disc was suspended by means of a PTFE basket in a jacketed vessel containing 175 $cm^3$ of 0.04 M citrate-phosphate buffer at pH 3.0. The buffer solution was stirred by a PTFE-coated magnetic bar and maintained at constant temperature by the continuous circulation of water from a bath at 30°C through the vessel jacket. The apparatus was protected from light and the absorbance of the buffer solution in the range 185 to 300 nm determined at timed intervals using a double beam scanning U.V. spectrophotometer. Where absorbance readings were greater than 1.0, sample solutions were diluted accordingly. The incubating buffer was replaced with fresh solution after each absorbance reading.

## 4.6 Results and discussion

### (i) Appearance and texture of polyacrylamide gels

Polyacrylamide gels were generally transparent and of rubber-like consistency; increasing monomer concentration caused them to become more brittle and rigid. An improvement in mechanical strength was observed with increased crosslinking (Bis) up to approximately 7C; crosslinking to a greater extent resulted in increasingly opaque gels of poor mechanical strength. Turbidity was also observed in gels polymerized at less than 10°C. Opacity in gels polymerized using low initiator concentrations and temperatures has been reported previously [147,168].

Polyacrylamide gels became increasingly opaque as crosslinking was increased above 7C, 15C gels appeared white. This phenomenon is discussed below in section 4.6.iii.

### (ii) Electron microscopy of polyacrylamide gels

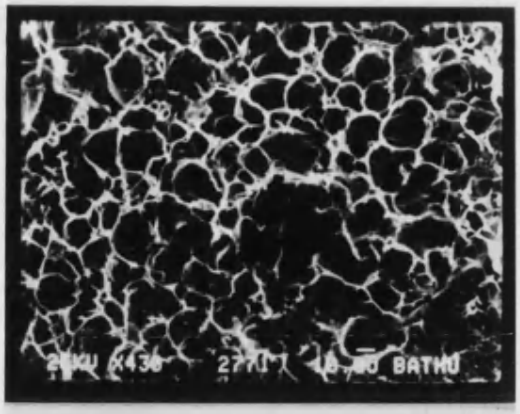
Electron micrographs shown in figures 4.3 to 4.5 illustrate the cellular construction of polyacrylamide gels of various formulations. A 5T 5C polyacrylamide gel such as used in rod gel electrophoresis (section 5) appears to have walls with thicknesses in the micrometer range, enclosing pores of 10 to 30 um diameter (figure 4.3.a.). Increasing gel concentration to 20T (5C) is seen to reduce pore size to generally less than 10 micrometers (figure 4.3.b.); heterogeneity in structure was commonly observed during electron microscopy studies of polyacrylamide gels. 20T 5C gels viewed at higher magnification (X4300) yielded no further information about the pore structure (figure 4.3.c.).

Figures 4.4.a. to 4.4.f. show the effect on polyacrylamide

FIGURE 4.3.a
ELECTRON MICROGRAPH OF 5T 5C
POLYACRYLAMIDE GEL

FIGURE 4.3.b.
ELECTRON MICROGRAPH OF 2OT 5C
POLYACRYLAMIDE GEL

FIGURE 4.3.c.
ELECTRON MICROGRAPH OF 2OT 5C
POLYACRYLAMIDE GEL


BATHU


25KV X430 2769 10 0U BATHU

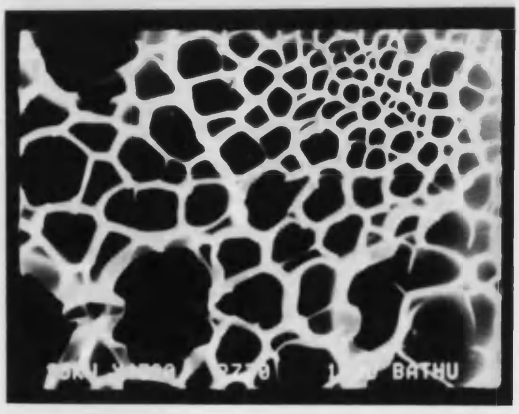

BATHU

FIGURE 4.4.a.

ELECTRON MICROGRAPH
OF 1OT 7C
POLYACRYLAMIDE GEL

FIGURE 4.4.b.

ELECTRON MICROGRAPH
OF 2OT 7C
POLYACRYLAMIDE GEL

FIGURE 4.4.c.

ELECTRON MICROGRAPH
OF 3OT 7C
POLYACRYLAMIDE GEL

FIGURE 4.4.d.

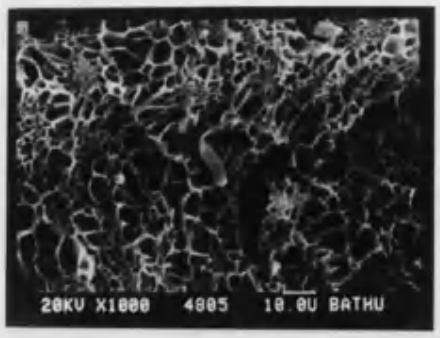
ELECTRON MICROGRAPH
OF 4OT 7C
POLYACRYLAMIDE GEL

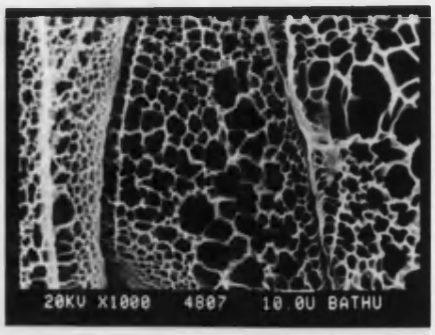
FIGURE 4.4.e.

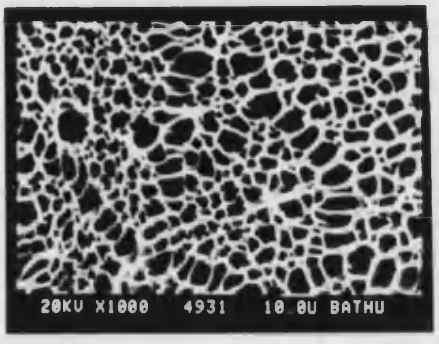
ELECTRON MICROGRAPH
OF 5OT 7C
POLYACRYLAMIDE GEL

FIGURE 4.4.f.

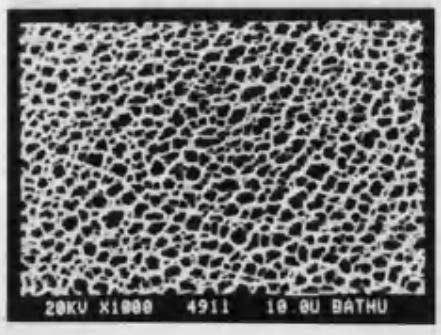
ELECTRON MICROGRAPH
OF 6OT 7C
POLYACRYLAMIDE GEL

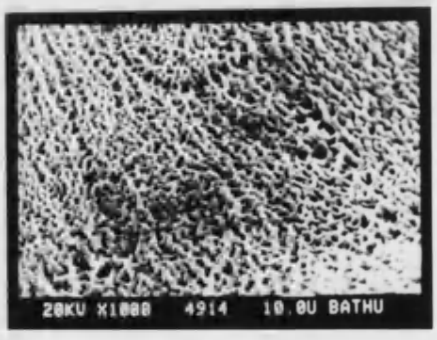

20KV X1000 4805 10.0U BATHU


20KV X1000 4807 10.0U BATHU


20KV X1000 4931 10.0U BATHU


20KV X1000 4811 10.0U BATHU


20KV X1000 4911 10.0U BATHU


20KV X1000 4914 10.0U BATHU

structure of increasing total gel monomer concentration in the range 10 to 60T (7C). A general increase in the concentration of mass per unit area is apparent in going from 10 to 60T; however an increase in wall thickness reported by Blank and Reimschuessel [170] with increased monomer concentraton was not found in this study. Cellular structure was difficult to make out in 60T polyacrylamide gels (figure 4.4.f.) even at higher magnification.

The influence on gel structure of crosslinking 30T polyacrylamide gel in the range 2 to 10C with Bis is shown in figures 4.5.a. to 4.5.f. Pores surrounded by clusters have been observed in 10C gels of lower (10T) total monomer concentration [170], clusters were not observed during this study.

It is considered that some wall thickening may occur when Bis is increased from 2 to 4C; subsequently, pore size becomes reduced (6,7 and 8C) with a slight increase in going from 8C to 10C.

The difficulty of interpreting electron microscopy data is highlighted by figure 4.5.d. where 7% crosslinked polyacrylamide gel appears to have a more open pore structure than 6C or 8C gels (figures 4.5.c. and 4.5.e.). Thus information derived from this technique is of limited usefulness since intrasample variation often exceeds that found between different gels. Sampling and sizing in a statistically valid and methodical manner may be a more appropriate technique to quantitatively study polyacrylamide gel structure by electron microscopy.

Nevertheless, some points are apparent from scanning electron microscopy of polyacrylamide gels. The cellular structures observed during this study, having diameters in the micron size range would

FIGURE 4.5.a.

ELECTRON MICROGRAPH
OF 3OT 2C
POLYACRYLAMIDE GEL

FIGURE 4.5.b.

ELECTRON MICROGRAPH
OF 3OT 4C
POLYACRYLAMIDE GEL

FIGURE 4.5.c.

ELECTRON MICROGRAPH
OF 3OT 6C
POLYACRYLAMIDE GEL

FIGURE 4.5.d.

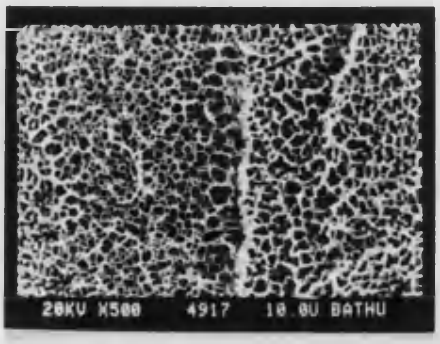
ELECTRON MICROGRAPH
OF 3OT 7C
POLYACRYLAMIDE GEL

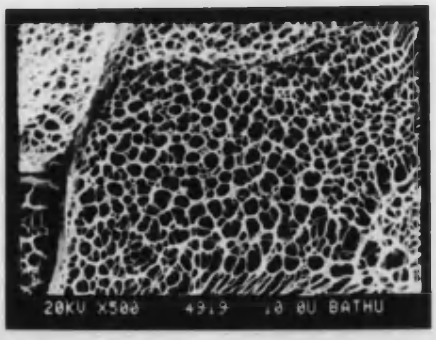
FIGURE 4.5.e.

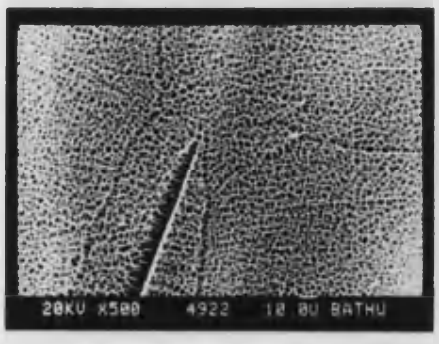
ELECTRON MICROGRAPH
OF 3OT 8C
POLYACRYLAMIDE GEL

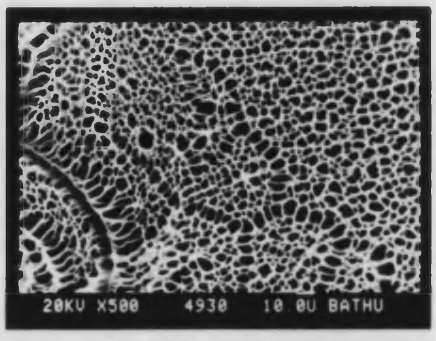
FIGURE 4.5.f.

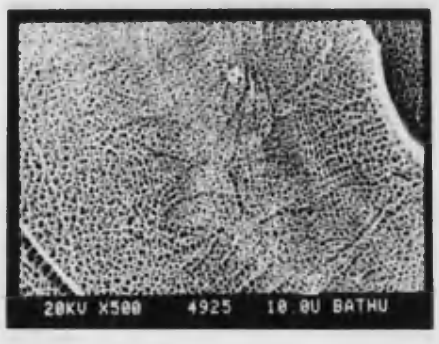
ELECTRON MICROGRAPH
OF 3OT 1OC
POLYACRYLAMIDE GEL

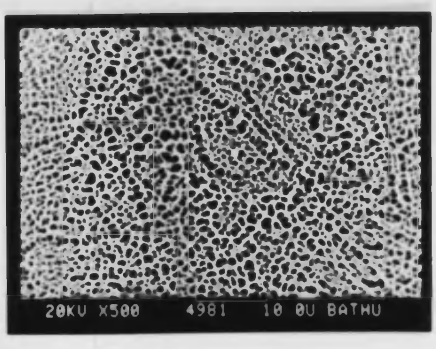

20KV X500 4917 10.0U BATHU


20KV X500 4919 10 0U BATHU


20KV X500 4922 10.0U BATHU


20KV X500 4930 10 0U BATHU


20KV X500 4925 10.0U BATHU


20KV X500 4981 10 0U BATHU

not produce a direct sieving action, even on macromolecules. The substructure of the surrounding walls is likely to be the source of molecular sieving found in these gels and will depend on the pores not being interconnected (section 1.5.4.1.2)this is in agreement with the hypothesis made by Calvert [173].

A cellular structure in the sub-micron range has been revealed by transmission electron microscopy of polyacrylamide gels [172].

### (iii) Swelling behaviour of polyacrylamide gels

#### (a) Ionic strength of immersing solution

Figure 4.6 indicates that the extent of polyacrylamide gel swelling increases with citrate-phosphate buffer ionic strength. Morris and Morris [163] also found that swelling of polyacrylamide gels was dependent upon buffer concentration. With gels carrying ionic groups (e.g. polyHEMA), the effect of ionic solutions are readily understood and have been well studied [7]. However, simple polyacrylamide gels are generally regarded as having no ionizable groups. A factor that may have potential effect is the affinity of the solute for the gel phase compared with that for the aqueous phase (partition coefficient). Boyde [174] studied the effect of pH (4-10) on hydrogel swelling and found gross polyacrylamide gel swelling at pH 9 and above.

#### (b) Polyacrylamide gel formulation

From figure 4.7, it can be seen that the degree of polyacrylamide gel swelling in both distilled water and citrate-phosphate buffer increased as total monomer concentration (T) increased; gels incubated in buffer showing a greater degree of

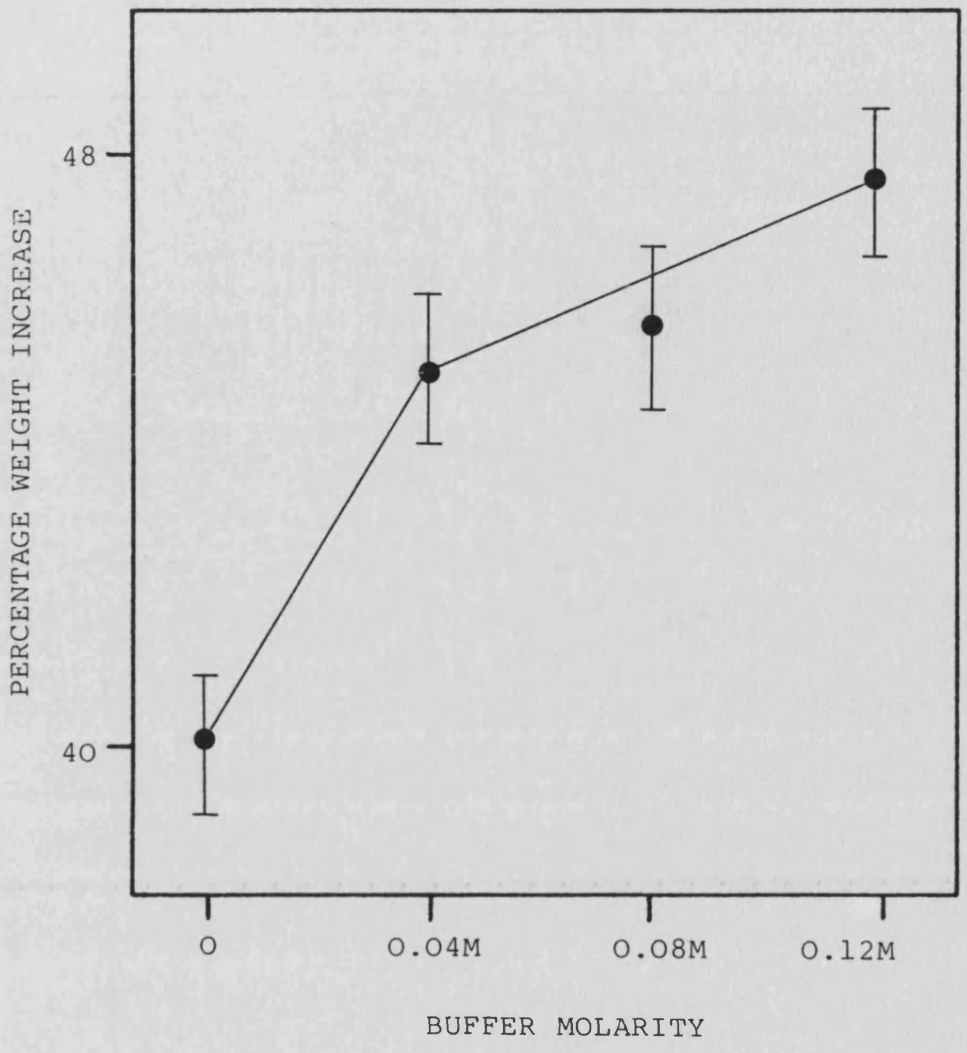



FIGURE 4.6 EFFECT OF CITRATE-PHOSPHATE BUFFER MOLARITY ON SWELLING OF 30T 7C POLYACRYLAMIDE GEL

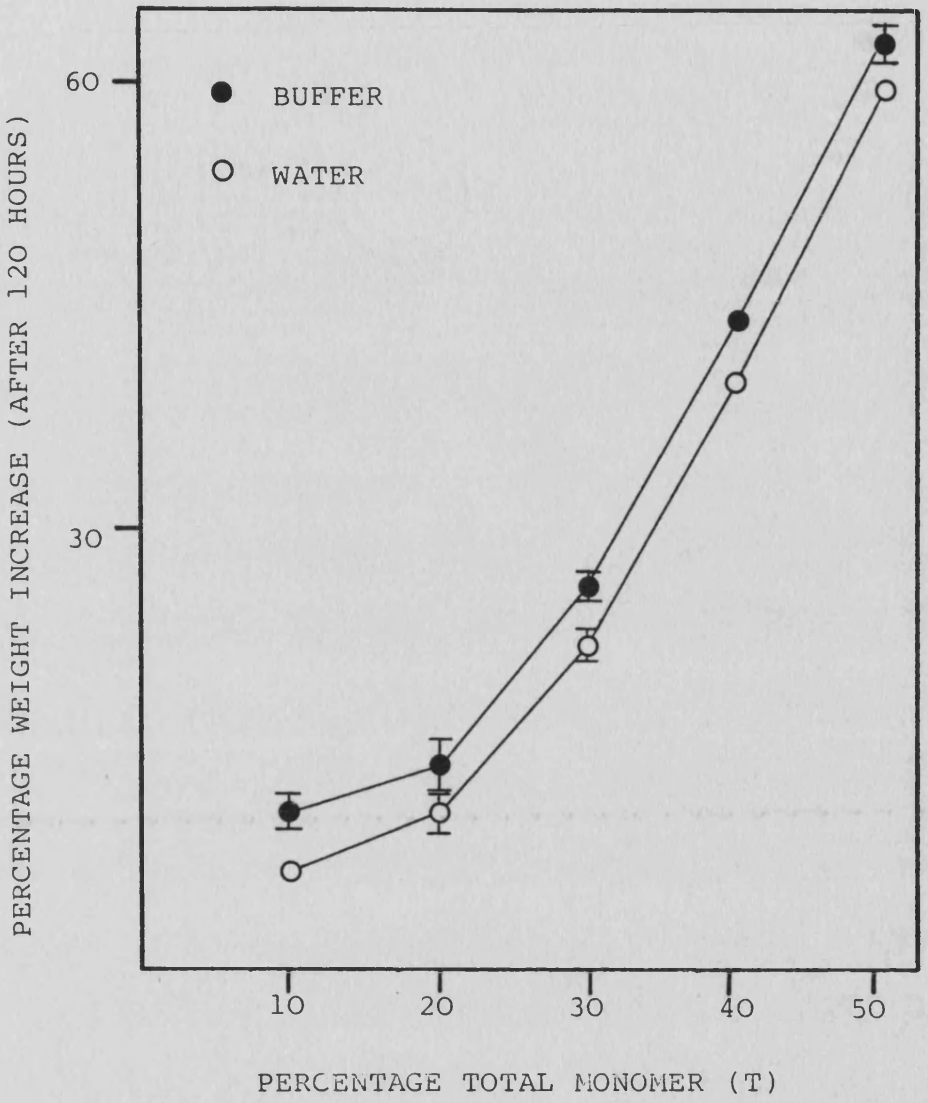



FIGURE 4.7 EFFECT OF TOTAL MONOMER CONCENTRATION (7C) ON SWELLING OF POLYACRYLAMIDE GEL IN 0.04M CITRATE-PHOSPHATE BUFFER AND DISTILLED WATER

swelling in all cases.

The above results are similar to those obtained by White and Dorion [204] who studied polyacrylamide gels in the range 5-50T and found that 5T and 10T gels showed almost identical swelling curves; Richards and Lecanidou [175] found a minimum of swelling at 9-15T. The greater concentration of polyacrylamide chains which have a tendency to become solvated (thus causing expansion of the hydrogel network) are probably responsible for the greater degree of swelling observed with increased total monomer concentration.

The degree of swelling in 30T polyacrylamide gels shows a marked fall (figure 4.8) between 2C and 10C. The extent of swelling is believed to be reduced by increasing C due to the reduced "contractility" of the gel network. 15C and 20C gels appeared to swell to a similar extent. This data shows good agreement with that predicted by Richards and Temple [176]. Hsu [168] suggests that above 10C, any additional Bis should, in an ideal crosslinked network increase crosslinking density but, in reality, forms clusters which leads to gel inhomogeneity. Richards and Temple [176] agree that increased local polymer concentration may lead to micro-syneresis (or phase separation) which accounts for polyacrylamide gel opacity at high crosslinking concentrations.

(c) **Effect of polymerization temperature**

Increasing the initial polymerization temperature from 10 to 30°C appears to increase the extent of polyacrylamide gel swelling (figure 4.9) but the relationship is not well defined. Gels polymerized at or below 10°C appeared opaque, thus it is possible that

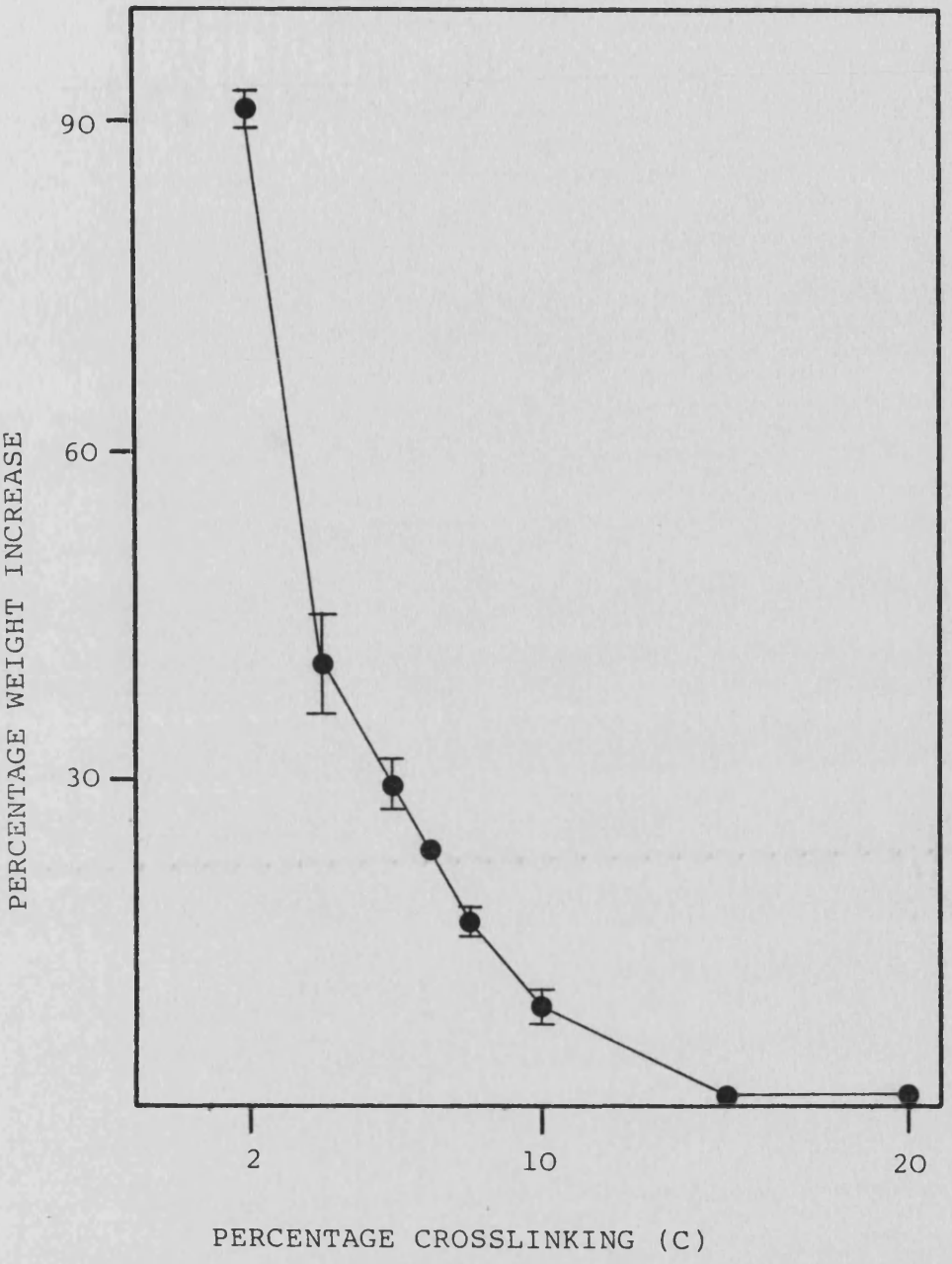



FIGURE 4.8 EFFECT OF CROSSLINKING ON SWELLING OF POLYACRYLAMIDE GEL (3OT) IN 0.04M CITRATE-PHOSPHATE BUFFER

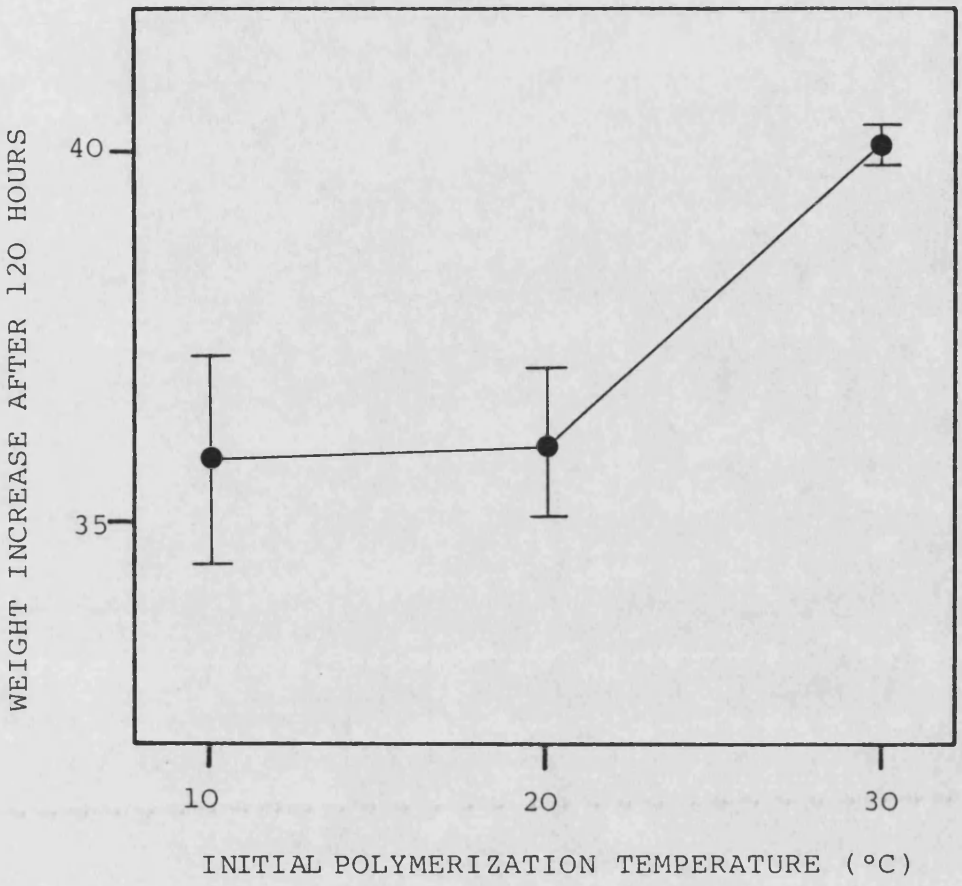



FIGURE 4.9 EFFECT OF POLYMERIZATION TEMPERATURE ON SWELLING OF 40T 7C POLYACRYLAMIDE GELS IN DISTILLED WATER

increased polymer "clustering" (see above) at lower polymerization temperatures leads to a decrease in effective crosslinking therefore reducing the capacity to swell.

(d) **Swelling during electrophoresis**

Electrophoresis does not appear to cause gel swelling to any marked extent; figure 4.10 shows that approximately a 1.5% gel weight increase is seen after 50 hours of electrophoresis using distilled water or citrate-phosphate buffer as electrolyte. The mechanism of any swelling caused by electrophoresis is unclear, although it is considered that electrolysis products may be involved.

On the basis of the results of polyacrylamide gel swelling studies, it was considered that gels should be stored in appropriate solutions for several days before use in electrophoretic release experiments so that equilibrium swelling was attained.

(iv) **Drug permeation and partitioning in polyacrylamide gels**

The difficulties encountered in trying to obtain drug partition coefficients in polyacrylamide gels are not easy to explain.

It is possible that microbial contamination may have caused interference. Buffer solutions were used in this study to simulate conditions during electrophoretic release studies, it is considered that the use of drug solutions in distilled water to immerse polyacrylamide gels for partition studies may yield more reproducible data.

From figure 4.11 it can be seen that drug permeabilities decreased with increasing polyacrylamide gel concentration. This is believed to be due to increased solute obstruction by the polymer

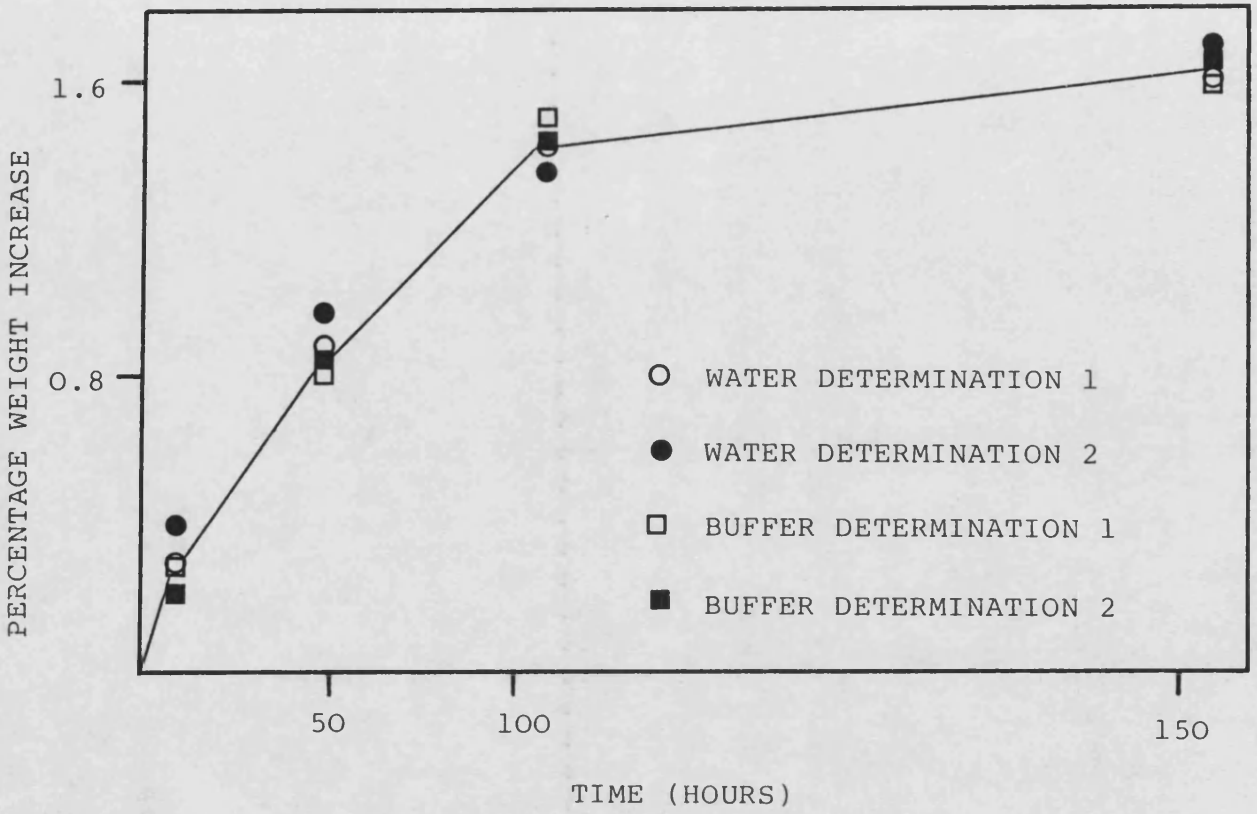



FIGURE 4.10 SWELLING OF 40T 7C POLYACRYLAMIDE GEL DURING ELECTROPHORESIS IN DISTILLED WATER AND CITRATE-PHOSPHATE BUFFER

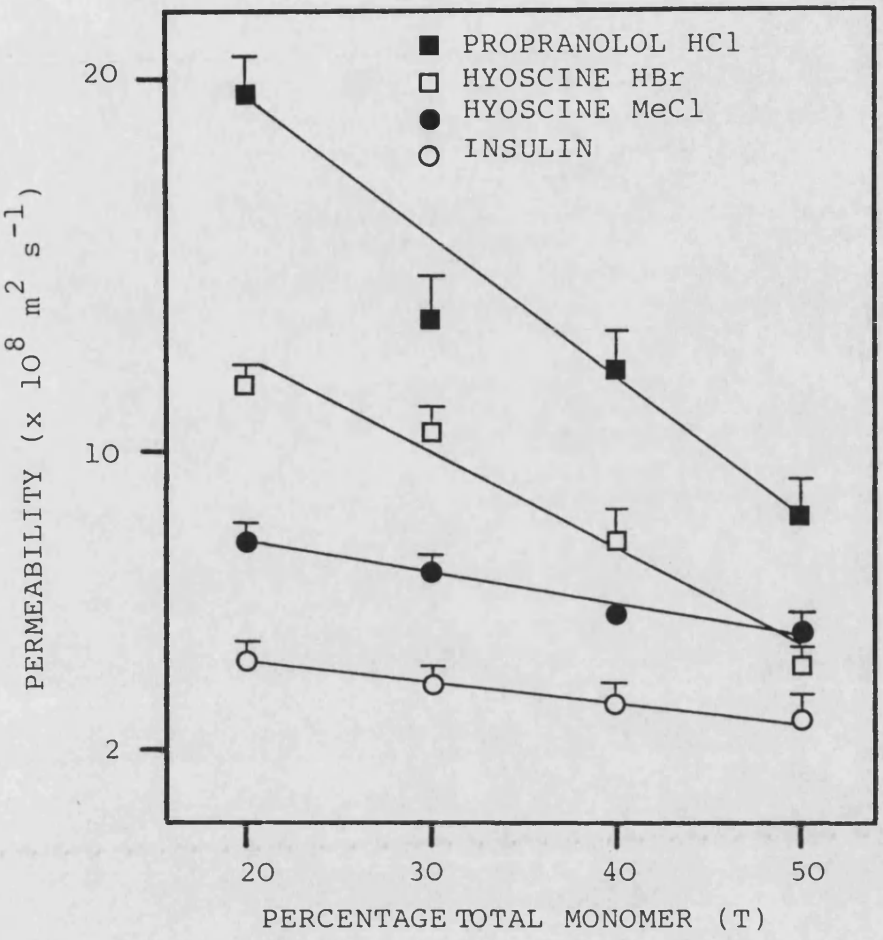



FIGURE 4.11 EFFECT OF TOTAL MONOMER CONCENTRATION (T) ON THE PERMEATION OF VARIOUS DRUGS THROUGH POLYACRYL-AMIDE GEL (7C)

network as T is increased. Relative permeabilities increased with decreasing (ionized) solute molecular weight in the order: insulin < hyoscine methyl chloride < hyoscine hydrobromide < propranolol hydrochloride. An exception was permeation in 5OT polyacrylamide gels where hyoscine methyl chloride showed a greater permeation rate than hyoscine hydrobromide. This data suggests that the above solutes migrated through the polyacrylamide gels tested via a "pore" mechanism which is dependent upon solute molecular volume and pore size of the polymer membrane [82].

Permeation of these solutes through higher polymer concentrations may occur by the "partition" mechanism (section 1.4.1.4.) e.g. as found by Wood et al. [107] who studied the diffusion of salicylic acid in polyHEMA gels.

Increasing the concentration of Bis was found to cause a reduction in the permeability of propranolol hydrochloride and insulin through polyacrylamide gels up to a crosslinking value of 7 to 8C (figure 4.12). (From gel chromatography experiments, Fawcett and Morris [205] found that at any given value of T, the permeability of proteins in polyacrylamide gels showed a minimum at about 5C). Above this concentration of crosslinker, permeation of both molecules was found to increase.

Increased solute diffusion rates through hydrogels of increasing crosslinking concentration have been reported [85,201] and were believed to be due to the increasing dominance of the "partition" mechanism over pore transport resulting from pore size reduction caused by increased crosslinking. Insulin is believed to diffuse by both mechanisms in hydrogels with an equilibrium hydration of 20%

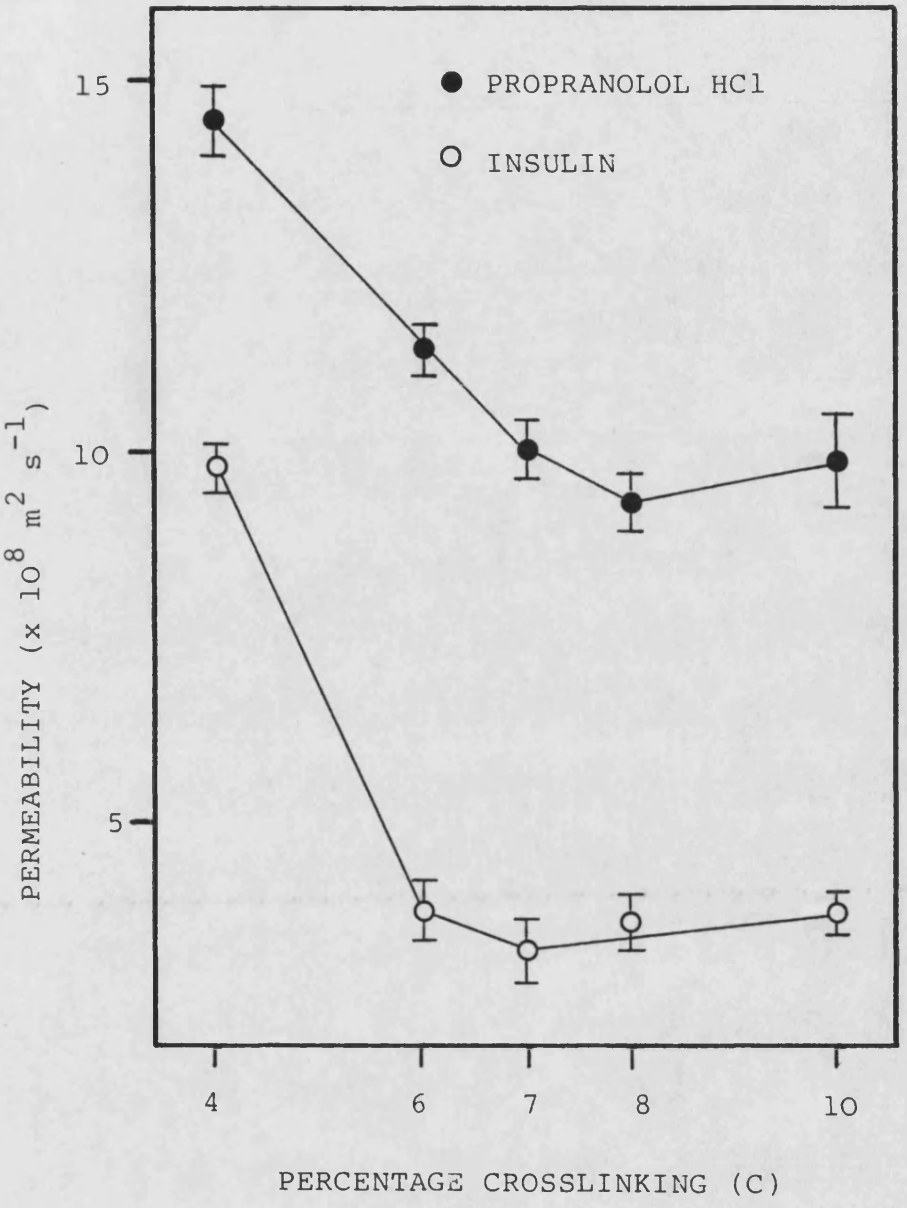



FIGURE 4.12 EFFECT OF CROSSLINKING ON PERMEABILITY OF PROPRANOLOL HCl AND INSULIN IN POLYACRYLAMIDE GEL (3OT)

[80]. In the present study which used highly hydrated (at least 50%) gels, the "pore" mechanism is considered to dominate at the crosslinker concentrations studied.

An increase in pore size is believed to account for increased solute permeability in polyacrylamide gels containing more than 7-8% crosslinker; a postulation supported by the results of electron microscopy of polyacrylamide gels (section 4.2.). However, in the absence of partition data, the possibility of drug transport through polyacrylamide gels by the partition mechanism cannot be excluded.

On the basis of drug permeability coefficients obtained from the above study, the fraction of drug likely to permeate in 4 days through 10 mm thickness 19mm diameter polyacrylamide gels during 2-compartmental electrophoretic release studies was calculated using equation 4.4. It was found that less than 0.3% of the original donor concentration of insulin would be expected to permeate into the receptor through 30T 7C polyacrylamide gel whilst less than 0.7% of hyoscine methyl chloride, hyoscine hydrobromide and propranolol hydrochloride would permeate through 40T 7C polyacrylamide gel during (a normal run time of) 4 days.

### (v) The removal of chemical impurities from polyacrylamide gel by incubation in buffer

The wavelength of maximum absorbance of the incubating solution was found to occur at 195 nm.

Figure 4.13 indicates that cumulative absorbance of the incubating buffer increased rapidly in the first 1-2 days of immersion followed by a steadily declining rate of increase thereafter. The nature of the leached material was not investigated but on the basis of the

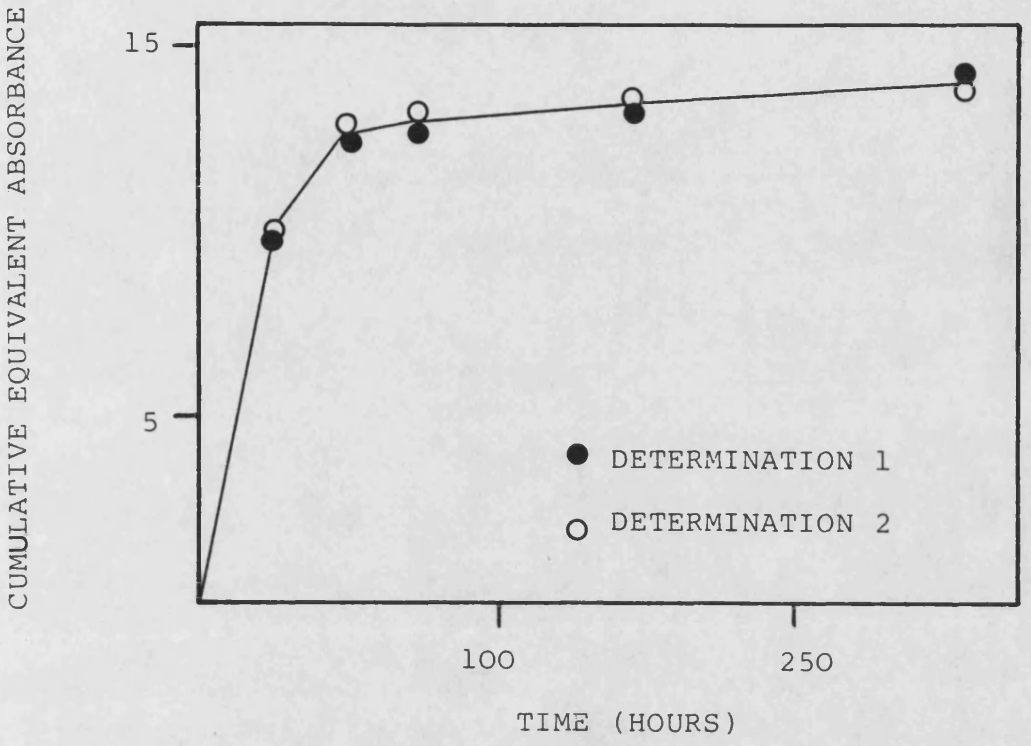



FIGURE 4.13 INCREASE IN UV ABSORBANCE OF A SOLUTION USED TO INCUBATE 4OT 7C POLYACRYLAMIDE GEL

absorbance maxima of the incubating solution (195 nm) was believed to be a mixture of unreacted monomers and catalysts used in gel polymerization. U.V. absorbing material has also been extracted from polyacrylamide gels by Loening [206] using a similar technique.

On the basis of results obtained in the above study, polyacrylamide gels were usually used only after several days of storage in buffer to ensure removal of most U.V. absorbing material.

# 5. ROD GEL ELECTROPHORESIS

## 5. Rod gel electrophoresis

The technique used in rod gel electrophoresis was based on widely described methods for zone polyacrylamide gel electrophoresis (section 1.5.4.), although voltages used for electrophoresis were generally much lower than usually employed in PAGE (section 1.5.4.4.).

### 5.1. Electrophoresis apparatus

Rod gel electrophoresis was performed using 2 types of apparatus: a commercial system (Model GE4, Pharmacia Fine Chemicals, Sweden) and a specially constructed device (figure 5.1). Both pieces of apparatus consisted of upper and lower electrolyte chambers of 0.5 and 1.5 $dm^3$ capacity respectively. 2 rows of 4 gel rod tubes were held precisely vertical in rubber grommets mounted in the base of the upper compartment in such a way that the gel within them connected the electrolyte reservoirs. Electrodes of platinum wire were located on the upper and lower edges of a central ridge in the upper vessel.

Stabilised power supplies generating constant voltages between 0 and 30 volts at 0 to 1 amp (E30/1, Farnell Instruments Ltd., Wetherby, Yorks) and 0 to 300 volts (EP3, Millipore) were used in conjunction with a multimeter to generate appropriate electric fields within the apparatus.

### 5.2. General technique of rod gel electrophoresis

5T 5C polyacrylamide rod gels (section 3.1.4.a.) were used unless otherwise stated. 0.5 $dm^3$ and 1.5 $dm^3$ of 0.025M Sorensen's phosphate buffer pH 7.4 was placed into the upper and lower electrolyte chambers respectively and the apparatus immersed (to the

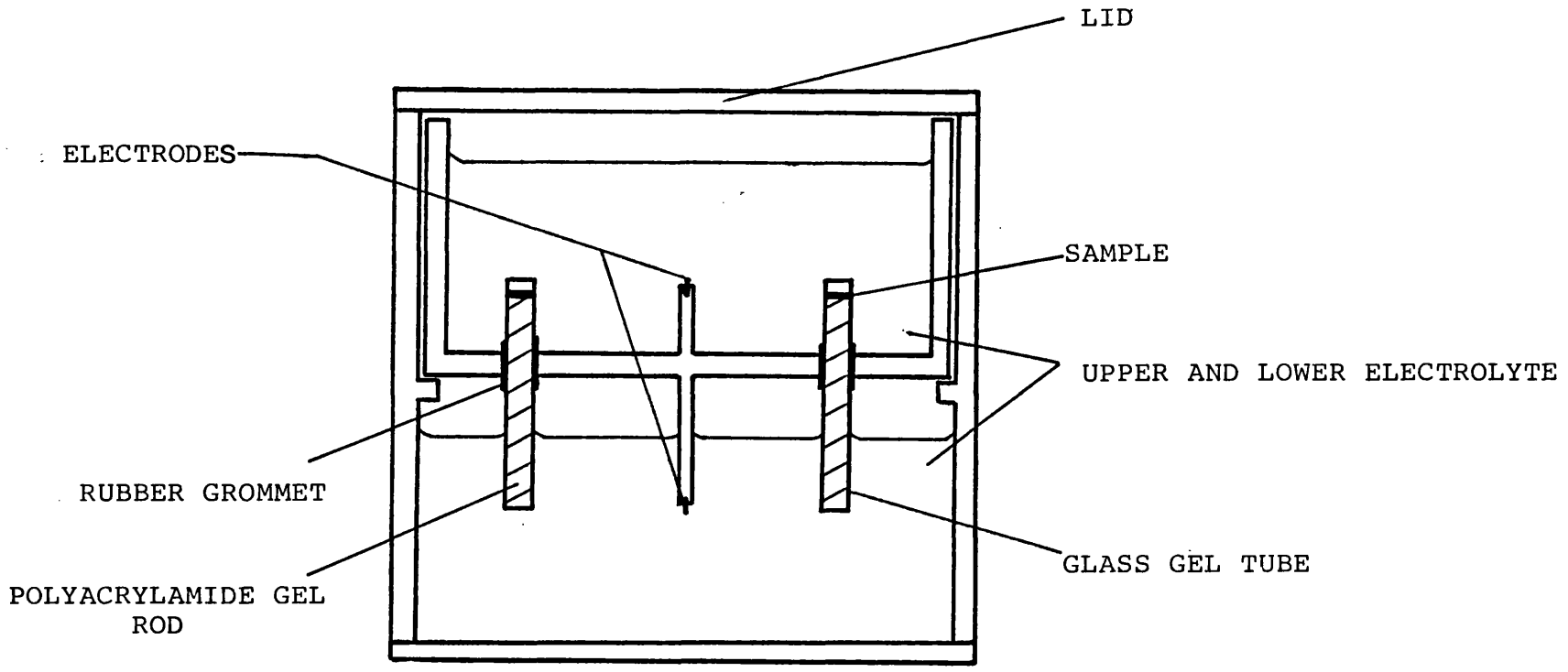



FIGURE 5.1 APPARATUS FOR ROD GEL ELECTROPHORESIS

level of electrolyte in the upper compartment) in a waterbath at $30 \pm 0.1^{\circ}C$. Pre-electrophoresis was performed at the proposed running voltage for 1 hour in an attempt to remove ammonium persulphate and other gel impurities.

A 50 $um^3$ sample of fluorescamine-labelled (Appendix 1) protein solution (equivalent to approximately $5 \times 10^{-5}$ g of protein) was carefully applied, by means of a replicating pipette (Gilson Pipetman P100), to the surface of each gel. The inclusion of glycerol (10% v/v) in the protein solution increased the density of the mixture and minimised dispersion of the sample after application.

Electrophoresis was commenced as soon as possible; the voltage and current were measured at the start and completion of each run. By use of a transparent water bath, the fluorescent protein bands could be observed migrating downwards. For each set of experiments, a run time was chosen to give sample migration distances to points approximately 1 to 2 cm from the bottom of each gel. Unless otherwise stated, a constant potential difference of 30 volts was applied. After termination of electrophoresis, gel tubes were removed from the apparatus and band migration distances accurately measured. Gels were examined against a black background using an ultraviolet light source to aid visualization of the fluorescamine-labelled proteins.

Protein band migration velocities were determined by dividing migration distance by electrophoresis time. 4 rod gels were used for each determination (figures 5.2, 5.3, 5.5 and 5.9 to 5.12) which was performed in triplicate; data points represent the mean of 3 determinations with vertical bars indicating standard deviation about the mean.

### 5.2.1. Factors affecting the electrophoretic migration velocity of proteins in polyacrylamide rod gel electrophoresis

(a) **Applied potential difference**

Voltages in the range 2 to 250 volts (0.2 to 35.0 mA) were employed to induce electric fields within the rod gel apparatus. This involved the use of 2 power supplies (0-30V and 0-300V). To assess the influence of using 2 different sources, experiments performed at 30 volts were repeated using both supplies. From the reproducibility of protein migration velocities obtained (figure 5.2), the procedure of utilising a more powerful, second power supply, for potential differences of 30V and above was considered satisfactory.

(b) **Electric current**

Currents in the range 0.2 to 35.0 mA (2 to 250 volts) were produced using two power sources. The validity of this method was assessed by a technique similar to that described above in section 5.2.1.a. and considered to be satisfactory. The current was found to fluctuate (usually decreasing during electrophoresis) e.g. from 0.29 to 0.26 mA and from 32 to 31 mA). The mean current was determined from readings obtained at the beginning and end of each run. The current per gel was obtained by dividing the mean current by the number of gels (usually 8) used in the rod gel apparatus.

(c) **Electrophoresis temperature**

The influence of temperature upon the migration velocity of insulin-F was determined by performing electrophoresis (at 30V) using temperatures in the range 15 to 50°C. For temperatures of 25°C and below, a cooling unit was necessary to maintain constant temperature

conditions. Changes in buffer pH were observed over the temperature range under study and where necessary, phosphoric acid or sodium hydroxide (1M) was added to adjust the buffer pH to 7.4.

(d) **Electrolyte ionic strength**

Sorensen's phosphate buffer was prepared and diluted to give solutions of ionic strengths in the range 0.001M to 0.5M. Where necessary, solutions were adjusted to pH 7.4 as described above in section 5.2.1.c. The influence of electrolyte ionic strength on the migration velocities of BSA-F and insulin-F was assessed by performing electrophoresis at a constant 30 volts.

(e) **The effect of electrolyte pH**

McIlvane's citrate-phosphate buffer in the pH range 3 to 8 was prepared. Potassium chloride was added to each solution as described by Elving et al. [207] producing buffers of constant ionic strength (0.5M). The attachment of fluorescamine (appendix 1) at amino groups interferes with the ability of a protein to act as a zwitterion. Thus to determine the influence of pH upon bovine serum albumin (BSA) and insulin electrophoretic migration, unlabelled proteins were utilised. These were visualised, subsequent to electrophoresis by a staining/destaining technique [208]:

1) Gels were removed from glass tubes by injection of water between gel and tube wall.
2) Each gel was placed into a test tube and "fixed" using a mixture comprising methanol: acetic acid: distilled water (5:1:5) for 1 hour.

3) Staining was allowed to proceed for 45 minutes in a protein stain, Coomasie Brilliant Blue R, dissolved in fixing solution to produce a 0.1% w/v solution.

4) Gel destaining, using a methanol: acetic acid: water (2:3:35) mixture was continued until protein bands were clearly visible in otherwise transparent gels (usually 30 to 60 minutes).

(f) **Percentage total monomer (%T) in gel**

Polyacrylamide rods in the concentration range 5 to 25% total monomer (T) 5% crosslinked (C) with Bis were used.

(g) **Extent of crosslinking (C) in gel**

5T polyacrylamide rods crosslinked to varying extents (2 to 30C) with Bis were used in this study.

**5.2.2. PolyHEMA rod gel electrophoresis**

This was performed using polyHEMA gels prepared by chemically initiated polymerization. 60, 70 and 80T gels, either crosslinked with EGDMA (0.5C) or uncrosslinked were employed. The rod gel electrophoresis apparatus and technique described in sections 5.1. and 5.2. were utilised for the study of BSA-F and insulin-F migration behaviour.

## 5.3 Results and discussion

### (i) Polyacrylamide rod gel electrophoresis

#### (a-b) Applied potential gradient and current

Protein sample bands obtained during rod PAGE were usually 1 to 2 mm in thickness. Occasionally, a fainter second band was observed; this was considered to be due to the presence of protein dimers.

Figures 5.2 and 5.3 show the effect of varying applied voltage and current respectively upon the electrophoretic migration of BSA-F and insulin-F in polyacrylamide gel (note log scales).

Curve 5.2 indicates a linear relationship between $\log$ protein migration velocities and log voltage applied between 10 and 250 volts. A linear plot was also obtained between log current per gel tube and $\log$ migration velocities of BSA-F and insulin-F in the range 0.1 to 1.6 mA (figure 5.3).

The steeper and non-linear portions of the curves in figures 5.2 and 5.3 are believed to be due to the effect of protein diffusion in polyacrylamide gels. Band broadening was observed after the longer run times required during lower electric field strength migration experiments. Thus it is considered that migration velocities recorded at lower field strengths were a result of protein movement due to both diffusion and electrophoresis; at 10 volts and above, the contribution of diffusion becomes minimal in comparison with electrophoretic migration. The linear plot of current (0.03 to 0.4 mA per gel rod) versus voltage in the range 3 to 15 volts shown in figure 5.4 suggests that no changes in system resistance occur around 10 volts (i.e. the voltage below which linearity was lost).

Insulin-F consistently migrated with greater velocity than BSA-F

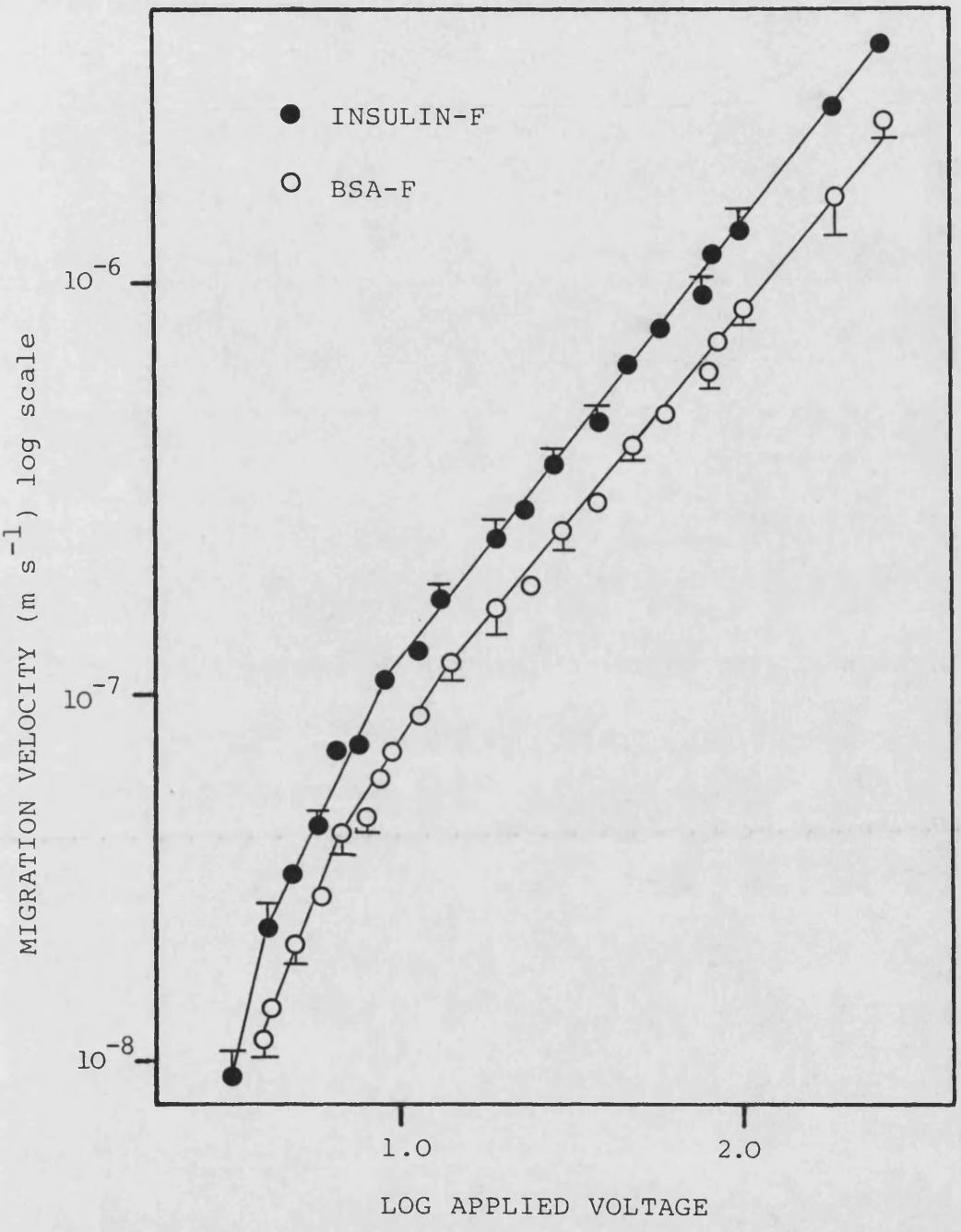



FIGURE 5.2 EFFECT OF APPLIED VOLTAGE ON MIGRATION VELOCITIES OF BSA-F AND INSULIN-F IN ROD POLYACRYLAMIDE GEL ELECTROPHORESIS

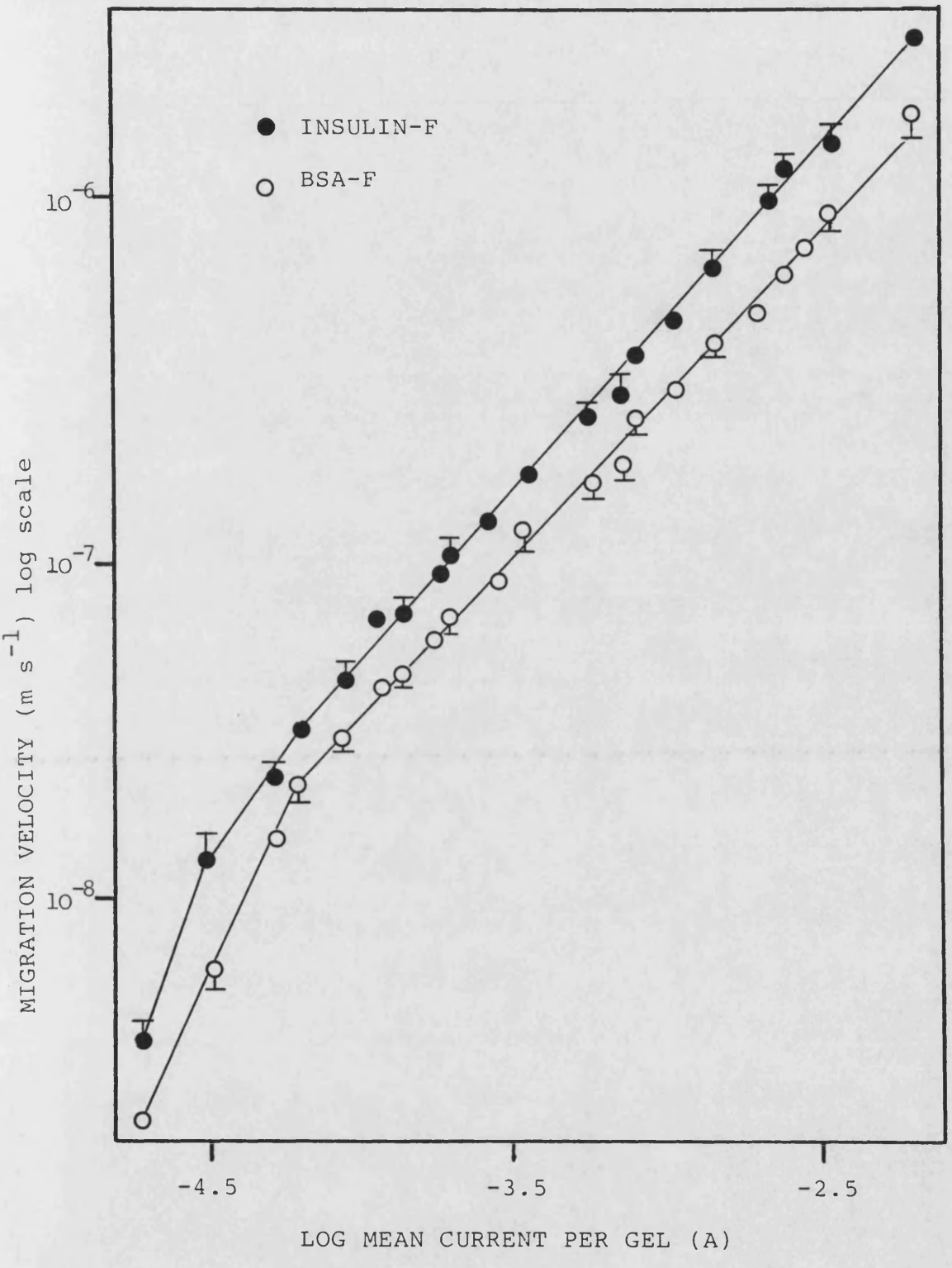



FIGURE 5.3 EFFECT OF CURRENT ON MIGRATION VELOCITIES OF BSA-F AND INSULIN-F IN ROD POLYACRYLAMIDE GEL ELECTROPHORESIS

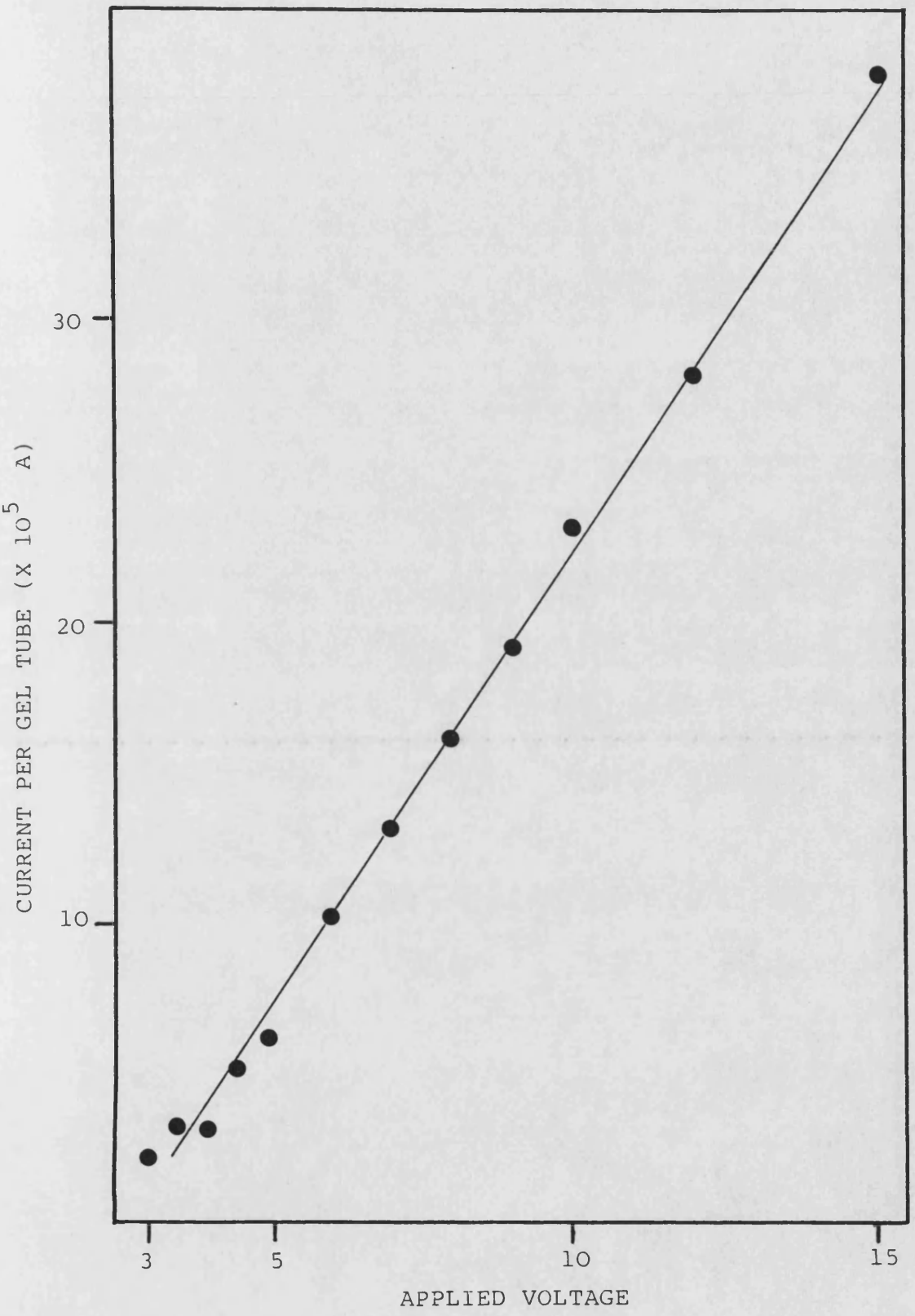



FIGURE 5.4 EFFECT OF APPLIED VOLTAGE ON CURRENT PER GEL TUBE IN POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

through polyacrylamide gels under similar conditions. This is not surprising since BSA has molecular weight an order of magnitude higher than insulin (i.e. $5.7 \times 10^4$ compared with $5.777 \times 10^3$). However the progress of molecules of less than $2 \times 10^4$ molecular weight such as insulin, has been found to be slower than expected on the basis of molecular weight considerations alone [209]. Although BSA is a much larger molecule than insulin, the rod-like shape of the former (15 x 3 nm) may enable it to migrate in a serpentine fashion (section 1.5.4.1.3.) thus giving migration velocities comparable with the insulin dimer (2 x 4 nm), the form predominating in solution [192].

In the absence of data relating to surface charge of BSA-F and insulin-F molecules, it is difficult to speculate about the influence of this factor on the migration velocities obtained.

For electrophoretic separations, it is normally advised that rod gel electrophoresis be performed using a minimum of approximately 100 volts or 1 mA per gel (section 1.5.4.4.iv.). Electrophoretic migration of BSA-F and insulin-F in 5T 5C polyacrylamide gel could not be detected below approximately 3 volts in the present study, although results indicate that low voltage (compared with conventional techniques) gel electrophoresis i.e. in the range 3.5 to 15 volts (0.03 to 0.4 mA per rod) is possible.

(c) **Temperature**

Figure 5.5 shows that the electrophoretic migration velocity of insulin-F (at a constant 30 volts) increased as temperature increased from 15 to 50°C; this relationship was as expected for the following reasons.

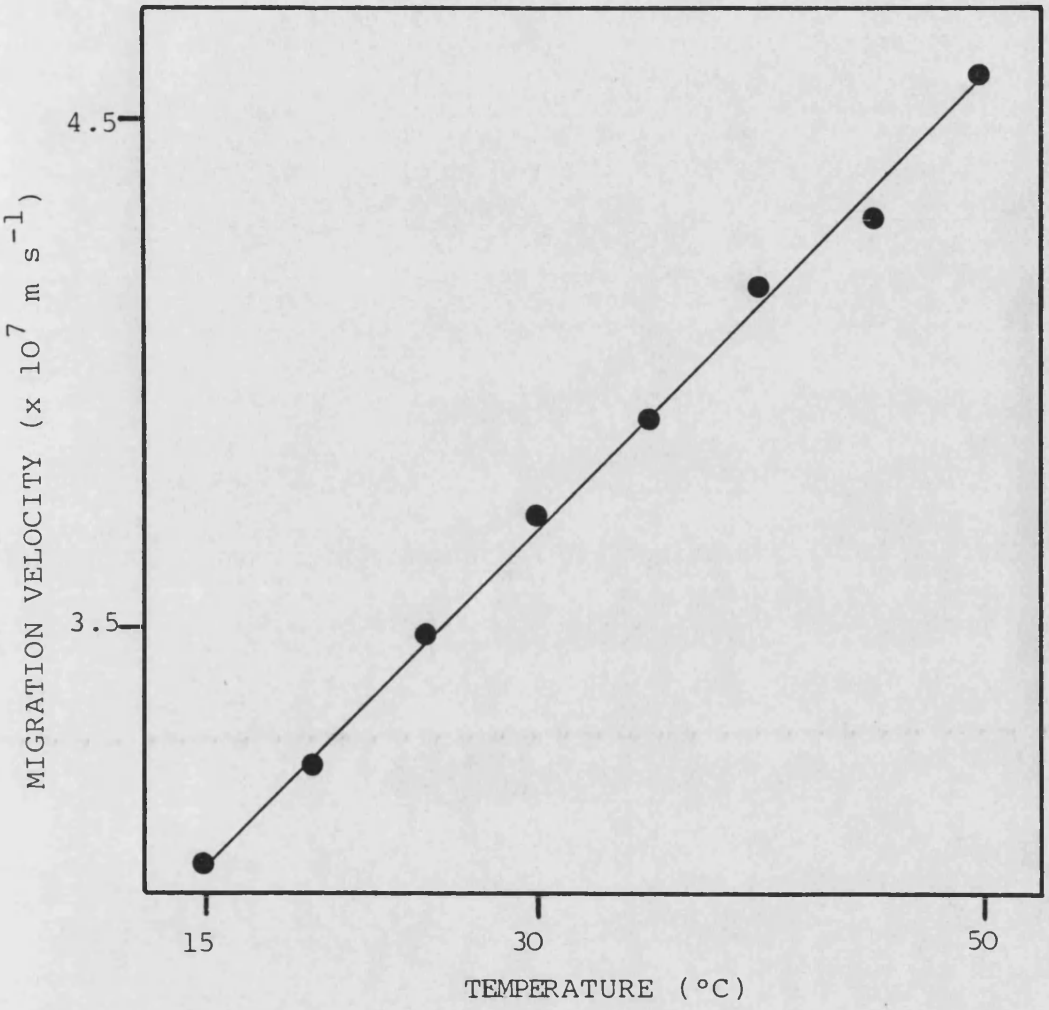



FIGURE 5.5 EFFECT OF TEMPERATURE ON INSULIN-F MIGRATION VELOCITY IN POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

Ionic mobility and free diffusion increase (the latter was observed as protein sample band broadening) with the raising of temperature because gel viscosity is reduced [135]. In addition, due to a decrease in gel and buffer electrical resistance, current was found to rise with increasing temperature (as shown in figure 5.6) which in turn may lower resistance. The above factors caused an overall increase in the insulin-F migration velocity of approximately 2% per degree rise of temperature; Morris and Morris [210] found that protein mobility increased by about 2.4% for the same temperature increase.

The effect of temperature on protein stability was not determined but due to the possibility of temperature related protein conformation changes as well as the points discussed above, it is clear that electrophoresis experiments should be carried out at constant temperature using a constant voltage or constant current power supply (see section 1.5.4.4.iv.).

(d) **Buffer electrolyte ionic strength**

The influence of buffer ionic strength on the migration velocities of BSA-F and insulin-F after electrophoresis at a constant 30 volts is shown in figure 5.7. It can be seen that as ionic strength was decreased from 0.5 M to 0.05 M, an increase in protein migration velocity was observed. However a further reduction in ionic strength from 0.05 M to 0.001 M resulted in decreased protein migration velocities. This is in contrast to the results of Gross [211] who found that mobility was approximately inversely proportional to the square root of the ionic strength. Absolom et al. [212] found that the electrophoretic mobility of BSA increased

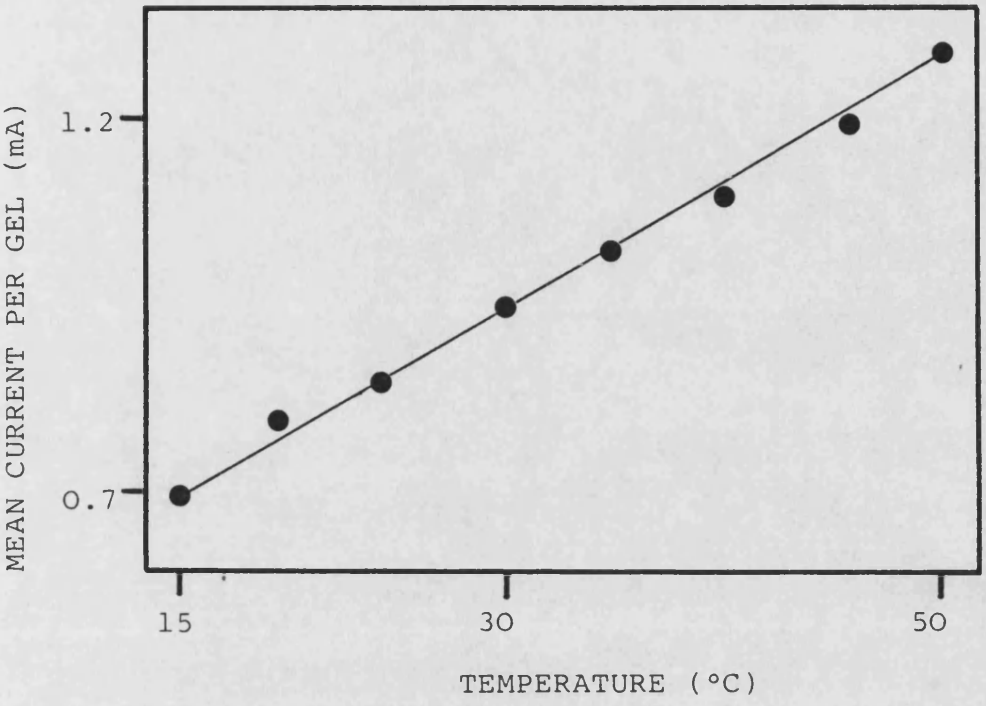



FIGURE 5.6 RELATIONSHIP BETWEEN ROD GEL ELECTRO-PHORESIS TEMPERATURE AND MEAN CURRENT PER GEL AT A CONSTANT 30 VOLTS)

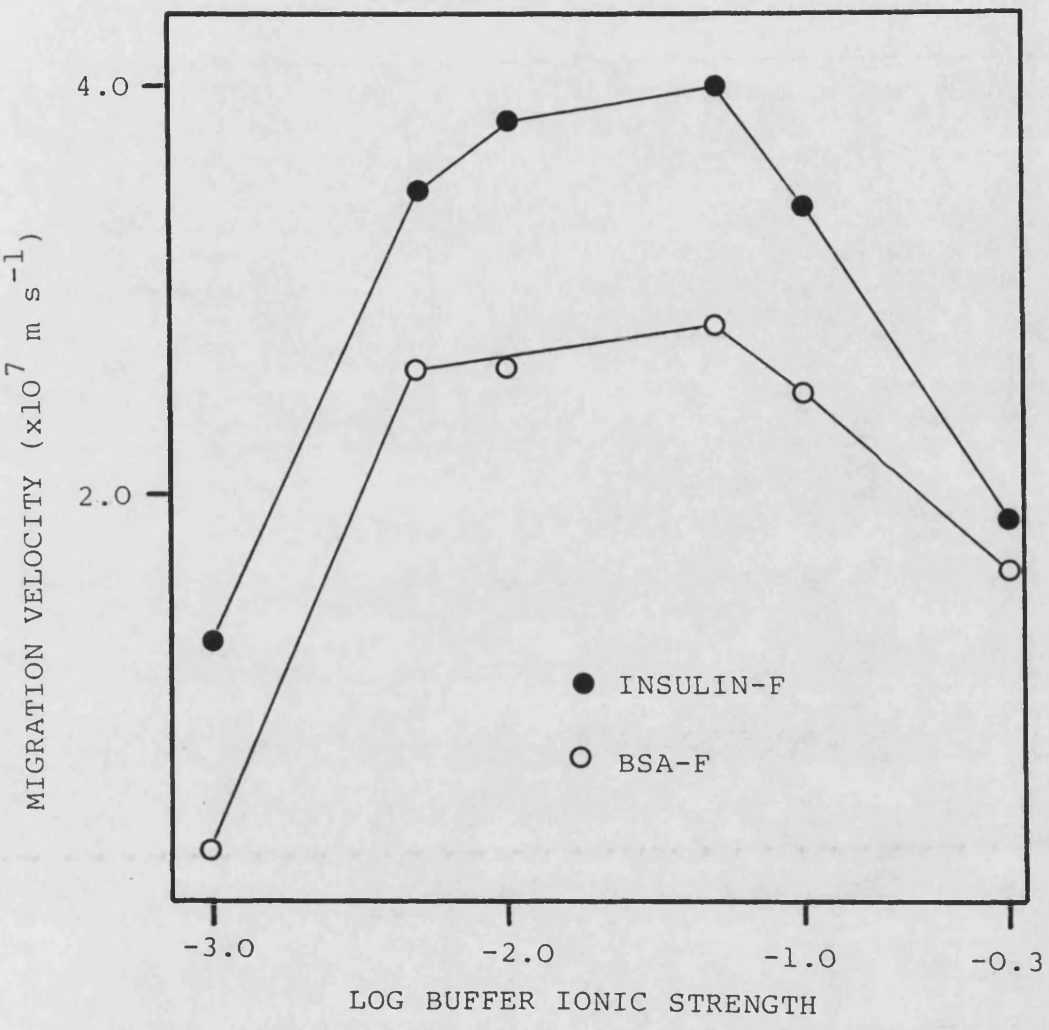



FIGURE 5.7 INFLUENCE OF BUFFER IONIC STRENGTH (pH 7.4) ON MIGRATION VELOCITIES OF INSULIN-F AND BSA-F IN POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

with a reduction in ionic strength but only ionic strengths in the range 0.01 to 0.05 M were investigated.

Insulin-F was found to migrate faster than BSA-F in all conditions. Sample bands became broader as buffer ionic strength was reduced; this has also been noted by Maurer [135] but not explained.

The above results are inconsistent with electrophoretic theory which states that electrophoretic mobility decreases with increasing ionic strength (1.5.4.4.iii.). However, the findings of this study may be explained by consideration of the effect of ionic strength upon another parameter in electrophoresis, the electric current. Figure 5.8 shows that current decreased as buffer ionic strength was lowered due to an increase in electrical resistance. The effect of current on protein migration is shown in figure 5.3. It is considered that the reduction in migration velocities caused by the decreasing current outweighs the expected increase in protein migration velocity with reduced ionic strength.

It was felt that the slight alteration of buffer ionic strengths caused by adjusting to pH 7.4 before use was justified in light of the influence of pH on protein migration velocities which is discussed below.

On the basis of the above results it was was decided that 0.025 M phosphate buffer would be most suitable for use as an electrolyte in 2-compartmental insulin electrophoretic release studies (section 6.3.) since high rates of migration using low voltages and/or currents was considered desirable.

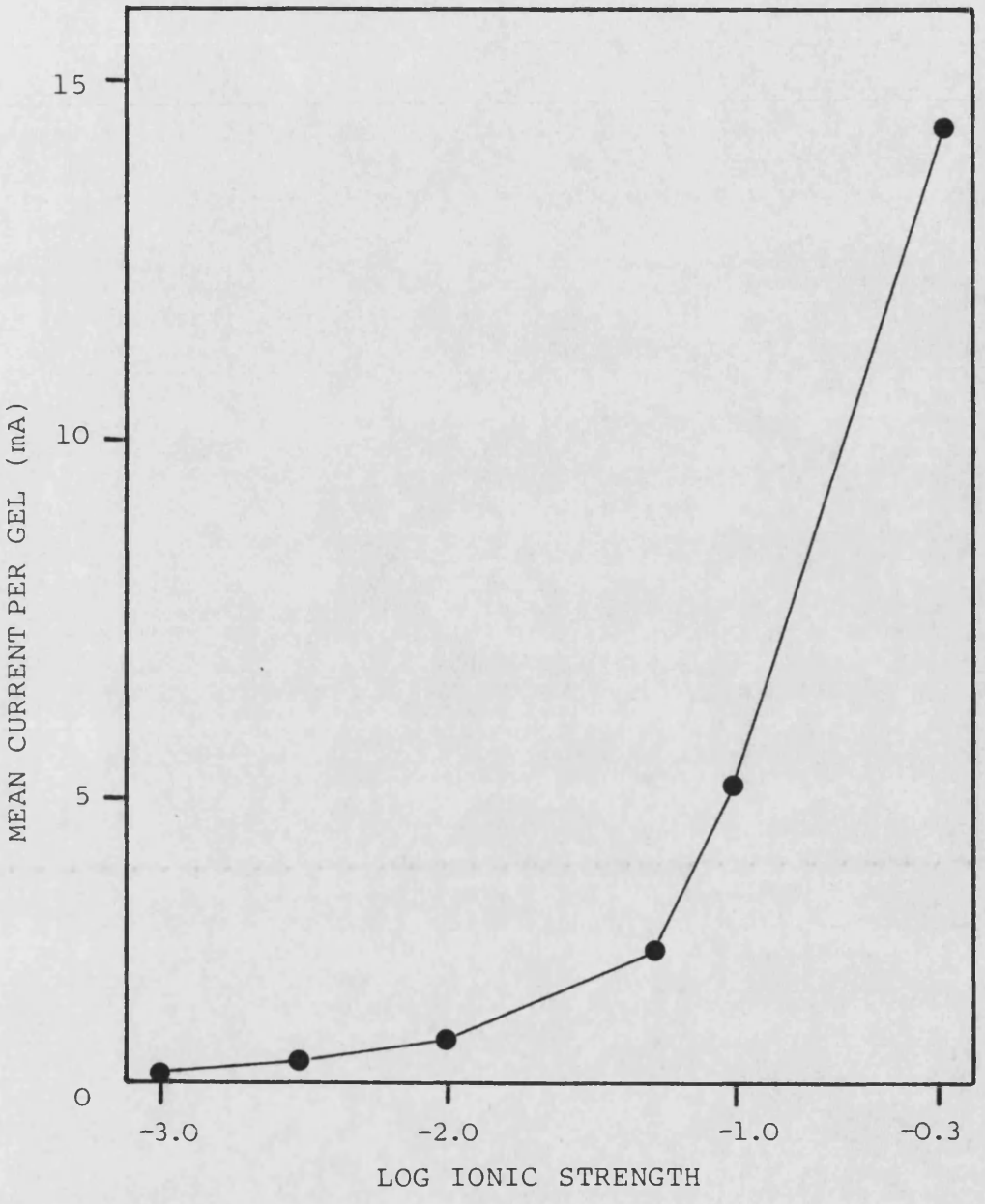



FIGURE 5.8 EFFECT OF BUFFER IONIC STRENGTH ON CURRENT IN ROD POLYACRYLAMIDE GEL ELECTROPHORESIS (AT A CONSTANT 30 VOLTS)

(e) **Electrolyte pH**

The Coomasie Blue staining procedure described in section 5.2.1.e. produced distinctive dark bands (representing BSA and insulin) in polyacrylamide gels.

Figure 5.9 indicates that the direction of protein migration was altered with changing pH. BSA appeared to show no net migration at pH 5.0 and by interpolation, insulin would be expected to remain stationary at about pH 5.5. These values represent the isoelectric points (pI) of the above proteins and show reasonable agreement with literature values (BSA = 5.1 and insulin = 5.7, section 2.1.1.). At the isoelectric point, the number of positively and negatively charged groups on a protein are balanced. As pH is increased, ionization of carboxyl groups predominates giving the molecules an increasing negative charge. Reduction of pH results in the increased ionization of amino groups resulting in a positively charged protein. The different isoelectric points and curve shapes (figure 5.9) obtained for BSA and insulin reflect the diversity in molecular structure of the 2 proteins. By convention, the sign of electrophoretic mobility is the same as that of the net charge of the protein [136].

Although PAGE is usually limited to the range pH 3 to 11 [154], it is considered that pH related effects in addition to ionization can affect electrophoretic mobility of proteins, by for example, producing degradation and conformational changes which may result in burying or exposure of ionizable groups.

The migration velocities of unlabelled BSA and insulin were comparable with those obtained using BSA-F and insulin-F during this study indicating that attachment of fluorescamine to the above

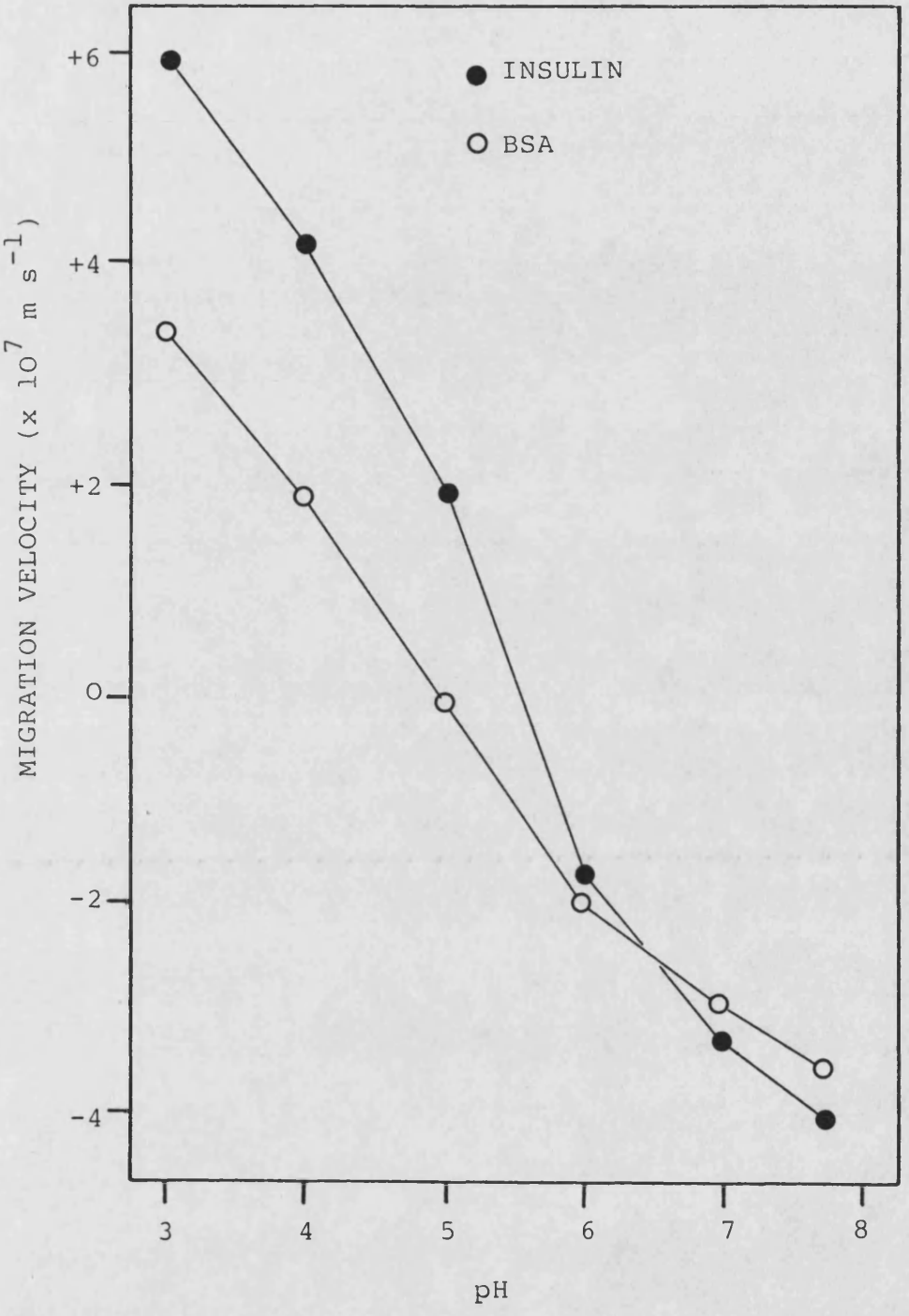



FIGURE 5.9 EFFECT OF BUFFER ELECTROLYTE pH ON MIGRATION VELOCITIES OF BSA AND INSULIN IN ROD POLYACRYLAMIDE GEL ELECTROPHORESIS

protein does not induce major change in the structures of BSA or insulin.

This investigation illustrates the importance of adequate buffering during polyacrylamide gel electrophoresis of proteins.

(f) **Percentage total monomer in gel**

Figure 5.10 shows the electrophoretic migration velocities of insulin-F and BSA-F in polyacrylamide gels of various total monomer concentrations (T); the migration velocities of both proteins decreased with increasing T. This is probably due to decreasing polyacrylamide gel porosity as monomer concentration is increased (section 1.5.4.1.1.). Insulin exhibits a linear relationship between migration velocity and T in the range 10 to 25T; the deviation from linearity below 10T is considered to be due to protein diffusion combining with electrophoresis to produce higher migration velocities than expected. Protein diffusion (insulin-F and BSA-F) was found to increase with decreasing T in polyacrylamide gels as evidenced by band broadening as monomer concentration was reduced.

BSA-F shows a different curve to that obtained for insulin, a marked reduction in migration velocity was observed as T increased from 5 to 15T with a smaller decrease noted between 15 and 25T. It is considered that the larger BSA molecule (m.w. $5.7 \times 10^4$) becomes increasingly sterically hindered compared with insulin (m.w. $5.777 \times 10^3$) as polyacrylamide gel porosity decreases (with increasing T). At 20-25T, a limiting pore size is approached (section 1.5.4.1.2.) and passage of the BSA-F molecule becomes increasingly difficult.

A linear relationship between log protein mobility (or log

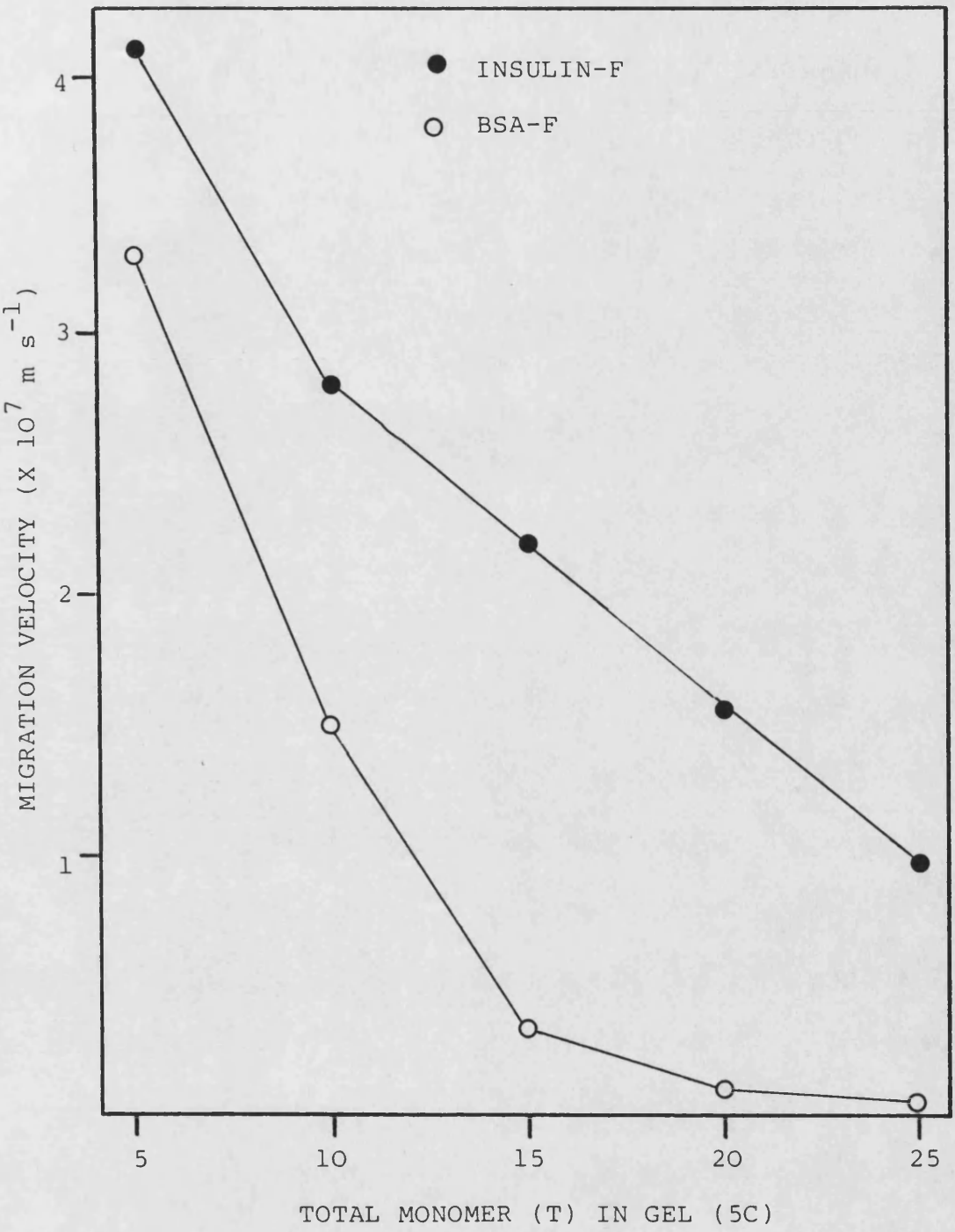



FIGURE 5.10 EFFECT OF TOTAL MONOMER (T) ON MIGRATION VELOCITIES OF BSA-F AND INSULIN-F IN POLY-ACRYLAMIDE ROD GEL ELECTROPHORESIS

relative mobility) and T is generally accepted and was first demonstrated in polyacrylamide gels by Hedrick and Smith [156]; this type of curve is used in the estimation of protein molecular weights (see section 1.5.4.3.a). Figure 5.11 shows the relationship between log migration velocities of BSA-F and insulin-F and T in polyacrylamide gels; both proteins show linear relationships which is in agreement with the general findings of Hedrick and Smith [156].

(g) **Crosslinking**

The influence of crosslinking (C) with Bis on the electrophoretic migration velocities of BSA-F and insulin-F through polyacrylamide gels is shown in figure 5.12. With both proteins, increased crosslinking caused an initial reduction in migration velocities which reached minimum values at 6-8C; then as Bis was increased from 8 to 30C, migration velocity increased. This is in agreement with the results of Rodbard et al. who investigated the effect of Bis using 7.5T polyacrylamide gels containing differing amounts of crosslinker and found that the mobilities of a number of proteins (including BSA) exhibited a minimum at 5-8C [167]. Thorun [213] demonstrated a linear relationship between log mobility and the square root of crosslinking in polyacrylamide gels but only made determinations in the range 0.1 to 5C.

On the basis of the above results and those found from insulin permeation experiments (section 4.6.), it is considered that changes in polyacrylamide gel pore size with increasing Bis concentration are reponsible for the observed effects on protein migration velocities; it is believed that after initially decreasing in size as crosslinking is increased up to 6-8C, aggregation of polymer chains

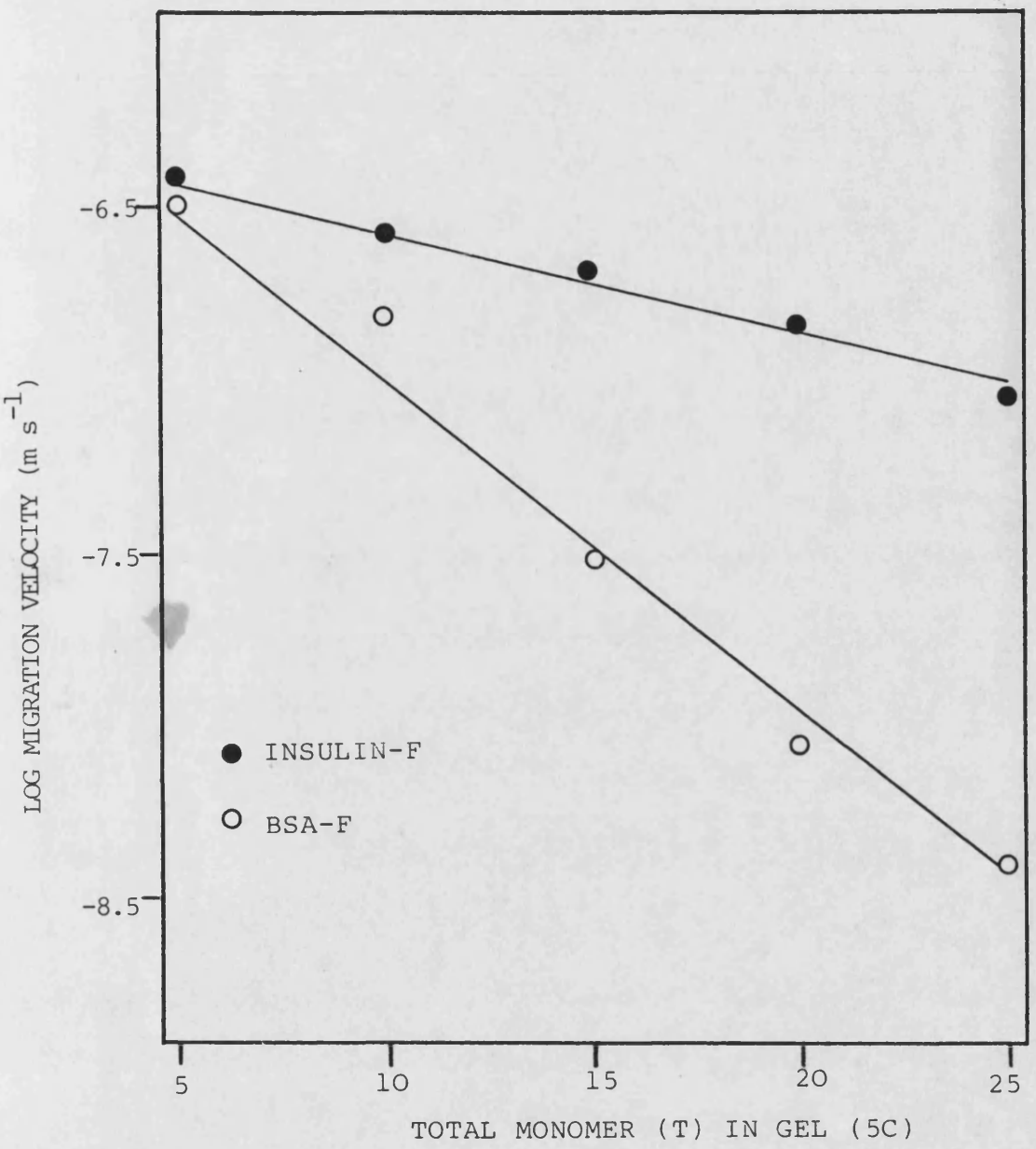



FIGURE 5.11 TOTAL MONOMER (T) VERSUS LOG MIGRATION VELOCITIES OF BSA-F AND INSULIN-F IN POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

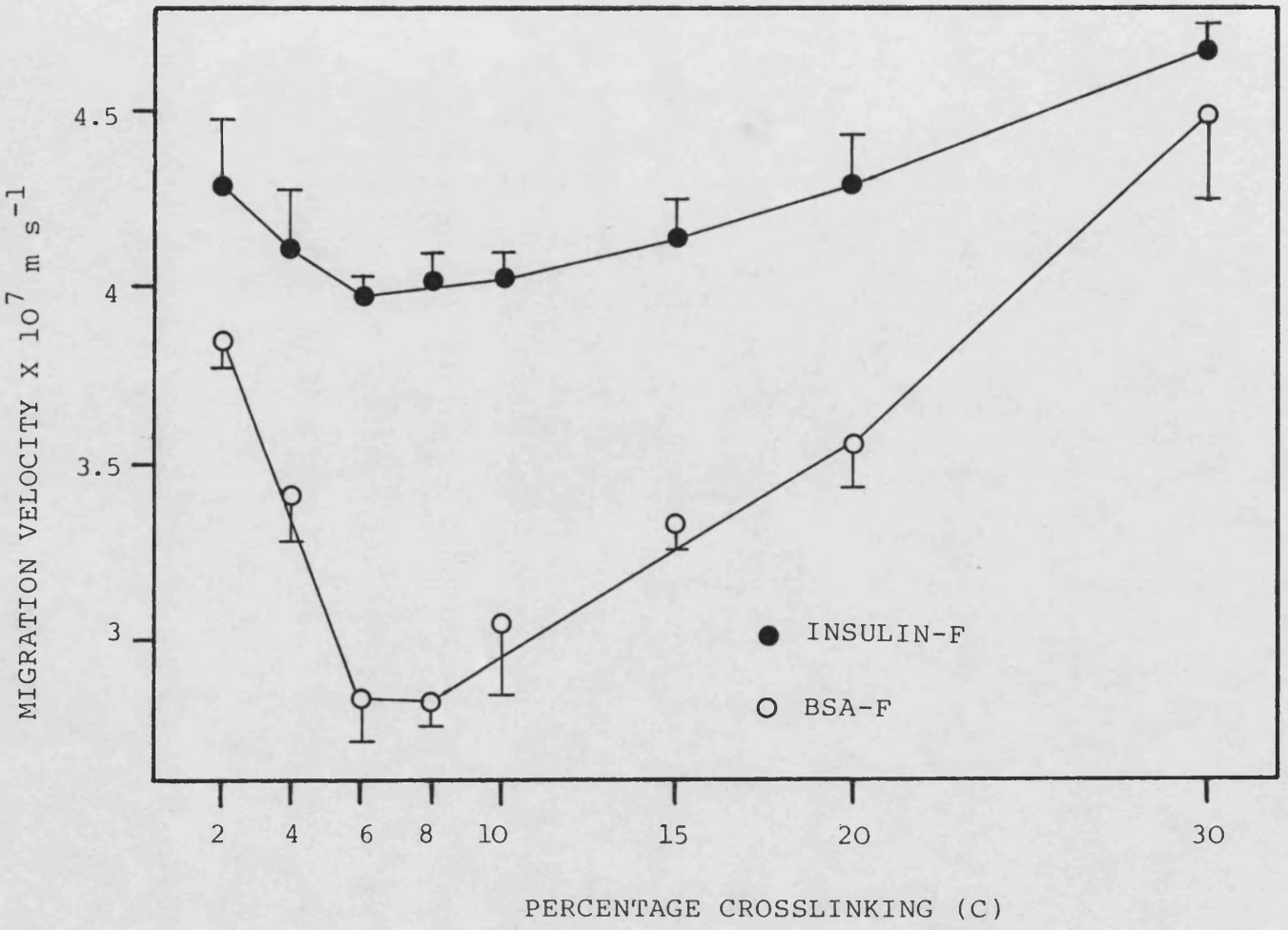



FIGURE 5.12 EFFECT OF CROSSLINKING (AT 5T) ON MIGRATION VELOCITIES OF BSA-F AND INSULIN-F IN ROD POLYACRYLAMIDE GEL ELECTROPHORESIS

bound together by the numerous crosslinkages results in larger pores as crosslinking increases above 8C. This "bundling" or "clustering" of polymer chains may account for the physical and optical properties of gels observed during the present study, i.e. polyacrylamide gels of 8C and above became increasingly opaque and mechanically weak. Fawcett and Morris [205] made similar observations about gel appearance and found that polyacrylamide gel pore size showed a minimum when C was about 5 per cent.

The migration velocity of BSA-F was reduced to a greater extent than that of insulin by increased gel crosslinking, probably because greater obstruction was experienced by the larger BSA-F molecules as pore size became reduced.

For the purposes of proposed 2-compartmental delivery studies (section 6), crosslinking in polyacrylamide gel should provide a minimal pore size to limit diffusion; on the basis of the above discussion, a value of 7C was selected.

(ii) **PolyHEMA rod gel electrophoresis**

It was found that neither BSA-F nor insulin-F migrated electrophoretically in polyHEMA gels of 60, 70 and 80 T (uncrosslinked and crosslinked with 0.5% EGDMA) to a detectable extent, even when a potential difference of up to 275 volts was applied. This was believed to be due to the smaller mean pore radius of polyHEMA gels, typically reported as 0.4 to 0.5 nm [85,214] for 60T gels compared with about 2-3 nm for 5T polyacrylamide gels (section 1.5.4.1.2.).

Although insulin permeability through polyHEMA (60T) membrane

has been demonstrated [200], it is considered that protein migration is prevented by uni-directional electrophoresis where molecules cannot escape from size limiting pores. In the permeation process however, insulin molecules are able to move in all directions and find an alternative route through the polymer network; a method for investigating the possible occurrence of this phenomenon might involve repeated electric field reversal during electrophoresis to help free protein molecules "trapped" within the gel pores.

Polyacrylamide gel was therefore utilised as the supporting medium for electrophoresis (in preference to polyHEMA) during the remainder of this study.

# 6. *IN VITRO* ELECTROPHORETIC DRUG RELEASE USING A 2-COMPARTMENT MODEL

## 6. *In vitro* electrophoretic drug release using a 2-compartment model

It was established in section 5 that a low voltage (5 to 30V) electrophoresis technique could be utilised in a reproducible manner to produce migration of BSA (F) and insulin (F) in polyacrylamide gel rods of various formulations. To assess the possibility of using physiologically acceptable, low voltage electrophoresis to produce controlled drug delivery, a 2-compartment apparatus (figure 6.1) was proposed.

It was intended that polyacrylamide gel should be used to form a virtual barrier to the permeation of drug from a reservoir. From permeation data described in section 4.6., it was calculated that polyacrylamide gel discs of 10 mm thickness and 19 mm diameter could be used to limit the permeation of insulin through 30T 7C gels, as well as hyoscine hydrobromide, hyoscine methyl chloride and propranolol hydrochloride through 40T 7C gels. An electric field generated by the use of low (5 to 20 V) voltages was then studied as as method for electrophoretically eluting drug from a reservoir through the polyacrylamide gel disc.

### 6.1. 2-compartment apparatus for electrophoretic drug release

Perspex of 0.6 cm thickness was employed in a specially constructed apparatus (figure 6.1).

Loss of insulin by adsorption onto glass and plastics is well known [193]. In this study, 95.2% and 94.7% of initial insulin concentration remained after 2 weeks storage at 30°C, away from light, of donor solutions (section 6.3.) stored in perspex and glass respectively. Donor solutions (section 6.4) of hyoscine hydrobromide, propranolol hydrochloride and hyoscine methyl chloride

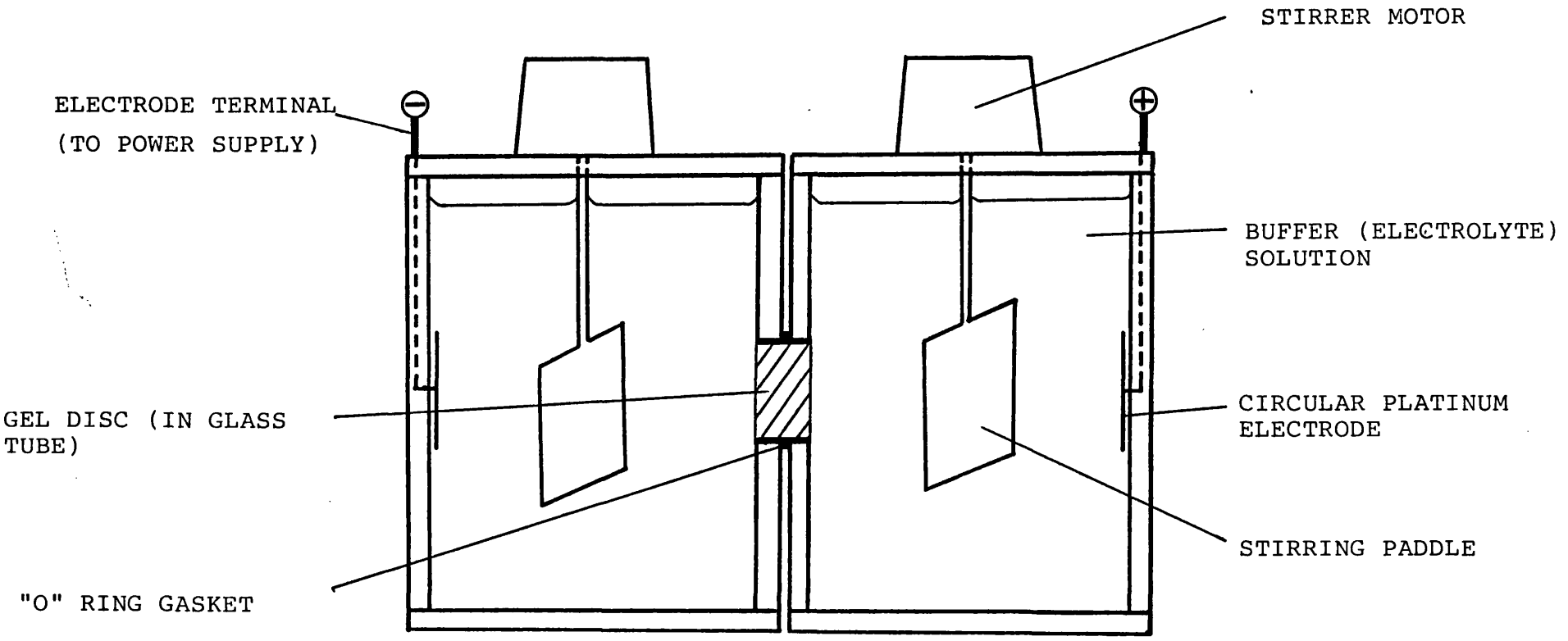



FIGURE 6.1 2-COMPARTMENT APPARATUS FOR ELECTROPHORETIC DRUG RELEASE

(at pH 3.0) showed no significant loss after similar treatment. The level of insulin loss in the perspex apparatus for electrophoretic drug release over a period of 3 days (normal experimental time) was expected to be approximately 1% and considered acceptable.

2 cube-shaped vessels measuring 6 cm x 6 cm x 6 cm formed the donor and receptor compartments. A polyacrylamide gel disc was placed between and connected the 2 compartments through an orifice of 2.25 cm diameter in the centre of one wall of each chamber. An "O" ring gasket was placed around the glass mould which enclosed the gel disc and ensured effective sealing of each compartment. When polyacrylamide gel membranes were used (sections 4.4.2. and 6.4.2.2.e.), they were simply clamped between the receptor and donor compartments.

A lid sealed each chamber and was screwed down securely. Small d.c. motors (Radio-Spares 336-321, RS Components Ltd., Corby, Northants, U.K.) were used to drive PTFE stirring paddles which revolved at 240 r.p.m. to ensure thorough mixing of donor and receptor solutions; to avoid solution frothing, a slower speed of 120 r.p.m. was used in studies involving insulin release.

An electric field, running perpendicular to the faces of the gel disc, was created by passing a current through the system by means of circular platinum electrodes embedded in the chamber walls. To minimise gas evolution at these electrodes, platinum wire used in their construction was treated in the following manner [186].

Electrodes were immersed in 50 $cm^3$ of chloroplatinic acid (BDH Chemicals) and platinum was electrodeposited on each electrode by passing a current of 10 mA through it for about 3 minutes after which

time the electrode surface became black.

Electrophoresis was performed at constant voltage using a stabilised power supply (WEIR 4000, Electroplan, Royston, Hertfordshire, U.K).

## 6.2 General method used in 2-compartment release studies

Polyacrylamide gel discs (of 19 mm diameter and 10 mm thickness) were removed from buffer solution used for storage and inspected to ensure flat surfaces and uniform composition. A gel disc (plus mould) was placed into the 2-compartment apparatus as described in section 6.1. and the effectiveness of the rubber gasket seal tested by filling one compartment with buffer solution; liquid seepage into the second compartment being indicative of ineffective sealing. After filling both chambers with buffer solution, pre-electrophoresis was performed for 1 hour at the proposed running voltage.

The apparatus was emptied and dried before being placed into a water bath maintained at $30 \pm 0.1^{o}C$. 175 $cm^3$ of donor (buffer containing drug) and receptor (drug-free buffer) solutions, equilibrated at $30^{o}C$ in the waterbath, were introduced into the appropriate compartments and the lids secured.

After stirring of solutions for approximately 1 minute, samples were removed from both compartments and electrophoresis commenced immediately afterwards. Subsequently, sampling was at timed intervals from the receptor solution to monitor the increase in drug concentration in that compartment. However, a sample was withdrawn from the donor solution at the end of each experiment to determine the extent of drug depletion in the donor compartment. When

necessary, donor and receptor volumes were replenished with the appropriate solution after sampling and the resulting dilution taken into account when analysing the release data. The voltage and current were measured regularly (using a multimeter) during the course of electrophoretic release studies, which were generally conducted for a period of 3 to 4 days.

Donor and receptor pH was measured at the start and end of most experiments.

Data is presented (figures 6.2 to 6.18) as receptor drug concentration (as a percentage fraction of the initial donor concentration) versus time. Each data point represents the mean of 2 determinations with vertical bars indicating experimental limits.

## 6.3. Electrophoretic release of insulin through polyacrylamide gel discs

Porcine insulin and radiolabelled drug was dissolved in 0.025M phosphate buffer, at pH 7.4, to produce a concentration of 2.85 I.U. $cm^{-3}$ (donor). Gentle stirring (by means of a P.T.F.E. coated magnetic bar) for several hours at room temperature was necessary to completely dissolve the protein and produce a clear solution; excessive agitation caused foaming of the solution and was avoided both during donor solution preparation and electrophoretic delivery experiments. Release of insulin by electrophoresis was assessed by monitoring increase in drug concentration in the receptor compartment.

### 6.3.1. Verification of electrophoretic insulin release by U.V. spectrophotometric assay

To ensure that insulin assay by gamma counting was not detecting a radiolabelled degradant of the hormone, insulin release due to electrophoresis using a voltage of 15 V and a current of $3\pm0.4$ mA through a 30T 7C polyacrylamide gel disc was determined by assaying receptor drug concentration by both U.V. spectrophotometry and gamma counting.

### 6.3.2. Factors affecting the electrophoretic release of insulin

a) **Applied potential difference**

Polyacrylamide gel discs of 30T 7C composition were employed to investigate electrophoretic insulin release at 0, 2.5 (0.3), 5 (0.7), 10 (2.0) and 15 (3.1) volts (mean current is shown in parentheses).

b) **Polyacrylamide gel concentration**

The release of insulin through 20, 25 and 30T polyacrylamide gels (all 7% crosslinked) at an applied potential gradient of 5 volts was determined.

c) **Alteration of electric field conditions during release**

To evaluate the feasibility of using electrophoresis to produce variable drug release rates, the release of insulin through 30T 7C polyacrylamide gel discs was monitored using cycling voltage levels. 5V and 10V potential differences were applied sequentially; in both cases the voltages were applied for approximately 24 hours each. The insulin release profile obtained when a 5 volt potential difference was repeatedly switched on and off (at 24 hour intervals) during release was also determined.

## 6.4. Electrophoretic release of hyoscine methyl chloride, propranolol hydrochloride and hyoscine hydrobromide through polyacrylamide gel discs

Factors affecting the electrophoretic release of insulin through polyacrylamide gel discs were studied in section 6.3.2. The possibility of using low voltage polyacrylamide gel electrophoresis (PAGE) to provide the controlled release of drugs of lower molecular weight was also assessed. Three drugs were selected for this purpose: hyoscine methyl chloride, propranolol hydrochloride and hyoscine hydrobromide (section 2.1.1.). Although gel electrophoresis of low molecular weight drug molecules e.g. antibiotics has been described [56,166], low voltage polyacrylamide gel electrophoresis of the drugs used in the present study has not been reported previously.

To ensure complete ionization of drugs, i.e., the form most appropriate for electrophoresis, the use of a buffer (McIlvane's citrate-phosphate) at pH 3.0 was proposed.

### 6.4.1. Buffering capability of citrate-phosphate buffer during electrophoresis

Changes in donor and receptor electrolyte pH were observed during electrophoretic insulin delivery (see section 6.5. below) and it was therefore decided that the buffering capability of McIlvane's citrate-phosphate buffers of various ionic strengths (intended for use in delivery experiments involving hyoscine hydrobromide, propranolol hydrochloride and hyoscine methyl chloride) should be determined. A 2-compartmental electrophoretic delivery apparatus previously described (section 6.1. and figure 6.1) with 40T 7C polyacrylamide gel discs of 10 mm thickness was used. McIlvane's

citrate-phosphate buffer, pH 2.85, was prepared and diluted to give solutions of ionic strengths ranging from 1.6 x $10^{-3}$M to 1.6 x $10^{-1}$M. Dilution caused buffer pH to increase up to a maximum value of pH 3.3 for the 1.6 x $10^{-3}$ M solution. Drug-free buffer solutions were placed into the donor (anolytic) and receptor (catholytic) compartments of the apparatus and a mean current in the range 0.5 to 3.3 mA passed through the system at a constant 10 volts. After approximately 100 hours of pre-electrophoresis at 30±0.1°C, donor and receptor electrolyte pH's were determined. The changes in pH of buffer solutions are shown below in table 6.1.

| Buffer ionic strength (x $10^{-2}$) M | Change in pH after pre-electrophoresis | | Mean current during pre-electrophoresis |
|---|---|---|---|
| | Donor | Receptor | |
| 16 | -0.6 | +0.5 | 3.3 mA |
| 8 | -0.7 | +0.8 | 2.6 mA |
| 4 | -0.7 | +0.8 | 2.2 mA |
| 1.6 | -0.7 | +0.8 | 2.2 mA |
| 0.16 | -0.6 | +1.7 | 0.5 mA |

**Table 6.1 The influence of ionic strength on the effectiveness of McIlvane's citrate-phosphate buffer during pre-electrophoresis**

The change in donor solution pH during electrophoresis appears to be little affected by buffer ionic strength, and as expected (see section 1.5.4.4.v) decreased (by 0.6 to 0.7 pH units). However,

increasing the ionic strength of the receptor buffer resulted in a smaller pH change in that compartment. Although 0.04M (initially at pH 3.0) buffer allowed pH fluctuations that might be considered unacceptable, it was decided that it should be used in subsequent electrophoretic release studies involving hyoscine methyl chloride, propranolol hydrochloride and hyoscine hydrobromide, for the following reasons.

The above 3 drugs have pKa's in aqueous solution of at least 7.35 (at 30°C) and will therefore be completely ionized over the pH range 2.3 to 3.8; secondly, high ionic strength buffers should be avoided as they can give rise to excessive heat generation during electrophoresis (section 1.5.4.4.iv). Finally, a 0.04M citrate-phosphate buffer is comparable with the 0.025M ionic strength phosphate buffer employed during studies of electrophoretic release of insulin (section 6.3).

### 6.4.2. Electrophoretic release of hyoscine methyl chloride

#### 6.4.2.1. Verification of electrophoretic release of hyoscine methyl chloride by HPLC

Liquid scintillation counting was employed to monitor drug concentration in the receptor. The following procedure was performed to ensure that this assay was detecting hyoscine methyl chloride and not a radiolabelled degradant.

During the course of electrophoretic drug release at a constant 10 volts, receptor solution which, on the basis of scintillation counting, had a concentration of $1.4 \times 10^{-5}$ mol $dm^{-3}$ was assayed by HPLC (section 2.2.6.) along with a hyoscine methyl chloride standard solution of $1.4 \times 10^{-5}$ mol $dm^{-3}$.

### 6.4.2.2. Some factors affecting electrophoretic release of hyoscine methyl chloride

Radiolabelled donor solution was prepared by dissolving the drug and tritiated hyoscine methyl chloride in 0.04M citrate-phosphate buffer, pH 3.0, to produce a $2.82 \times 10^{-4}$ mol $dm^{-3}$ solution. Unless stated otherwise, 40T 7C polyacrylamide gel discs of 10 mm thickness and 19 mm diameter were employed.

a) **Applied voltage**

Hyoscine methyl chloride concentration in the receptor compartment was monitored at timed intervals whilst 0, 5 (1.5 mA), 10 (3 mA), 15 (5 mA) or 20 (8 mA) volts were applied to the 2-compartment delivery apparatus (mean current is shown in parentheses).

b) **Polyacrylamide gel concentration**

The electrophoretic release of hyoscine methyl chloride through polyacrylamide gels of 30, 35 and 40T (7C) composition was investigated at an applied potential difference of 10 volts.

c) **Alteration of electric field conditions during delivery**

The electrophoretic release of hyoscine methyl chloride through polyacrylamide gel discs was monitored whilst a potential difference of 10 volts applied across the delivery apparatus was either (i) repeatedly switched on and off at approximately 24 hour intervals, or (ii) maintained at a constant 10 volts.

d) **Temperature**

The effect of temperature on the electrophoretic release (at a potential difference of 10 volts) of hyoscine methyl chloride was assessed by performing delivery experiments in constant temperature waterbaths at: 25,30,35,40 and 45$^{o}$C.

e) **Polyacrylamide gel thickness**

40T 7C polyacrylamide gel sheets of 1.5, 5.5 and 10 mm thicknesses were prepared by a method previously described (section 3.1.4.c.). Discs of 35 mm diameter were stamped out from gel sheets using specially constructed cutting rings and clamped between donor and receptor compartments of the electrophoretic release apparatus. In the absence of glass moulds around gel discs the exposed polyacrylamide gel surface area in each compartment was increased from 2.8 to 4.0 $cm^2$, and this was taken into account during analysis of electrophoretic release data. After the initiation of electrophoretic drug release, sampling from the receptor solution was carried out at 60 minute intervals for the first 7 hours, with a final sample after 24 hours of delivery.

f) **Donor drug concentration**

Hyoscine methyl chloride was dissolved in 0.04M citrate-phosphate buffer to produce donor solutions of the following concentrations: $2.82 \times 10^{-2}$, $2.82 \times 10^{-3}$, $2.82 \times 10^{-4}$ and $2.82 \times 10^{-5}$ mol $dm^{-3}$. A potential difference of 10 volts was applied across the delivery apparatus.

g) **Duration of electrophoretic release**

To determine the limit of zero-order release kinetics with respect to time, electrophoretic delivery of hyoscine methyl chloride at a constant potential difference of 10 volts was continued for 10 days.

### 6.4.3. Electrophoretic release of propranolol hydrochloride and hyoscine hydrobromide through polyacrylamide gel discs

Drug donor solutions were prepared by dissolving hyoscine hydrobromide and propranolol hydrochloride in 0.04M citrate-phosphate buffer to produce $2.60 \times 10^{-4}$ and $3.38 \times 10^{-3}$ mol $dm^{-3}$ solutions respectively. 40T 7C polyacrylamide gel discs of 10 mm thickness and 19 mm diameter were used for release experiments.

#### 6.4.3.1. Effect of applied voltage on the electrophoretic release of hyoscine hydrobromide and propranolol hydrochloride

Potential differences of 5 and 10 volts were applied to the delivery apparatus and the concentration of propranolol hydrochloride and hyoscine hydrobromide in the receptor compartment monitored by HPLC assay (sections 2.2.4 and 2.2.5).

## 6.5. Results and discussion

### 6.5.1. Electrophoretic Insulin Release

#### (i) Donor drug loss during 2-compartment insulin release studies

Donor insulin concentrations decreased as receptor drug concentration increased during the course of electrophoretic release studies; approximately 2% (of the original donor concentration) of insulin usually remained unaccounted for at the end of experiments of 3 to 4 days duration. Drug loss was believed to be due to insulin retention within the polyacrylamide gel disc and by adsorption onto the walls of the electrophoresis apparatus (section 6.1).

#### (ii) Verification of electrophoretic insulin release by U.V. spectrophotometry

Figure 6.2 shows the receptor concentration of insulin determined by U.V. spectrophotometric and gamma counting assays. Although only 3 data points were used, data from the 2 assay procedures was comparable suggesting that the gamma counting assay method was probably detecting insulin and not a radiolabelled degradant. Techniques such as HPLC and radiommunoassay may yield more information regarding insulin stability but were not used in the present study.

The slope of the curve in figure 6.2 indicates an insulin release rate of 15.4 I.U. per day.

#### (iii) Factors affecting electrophoretic release of insulin

##### (a) Applied potential gradient (electric field strength)

In figure 6.3 the effect of applied voltage on the electrophoretic release of insulin is shown. At zero volts, insulin

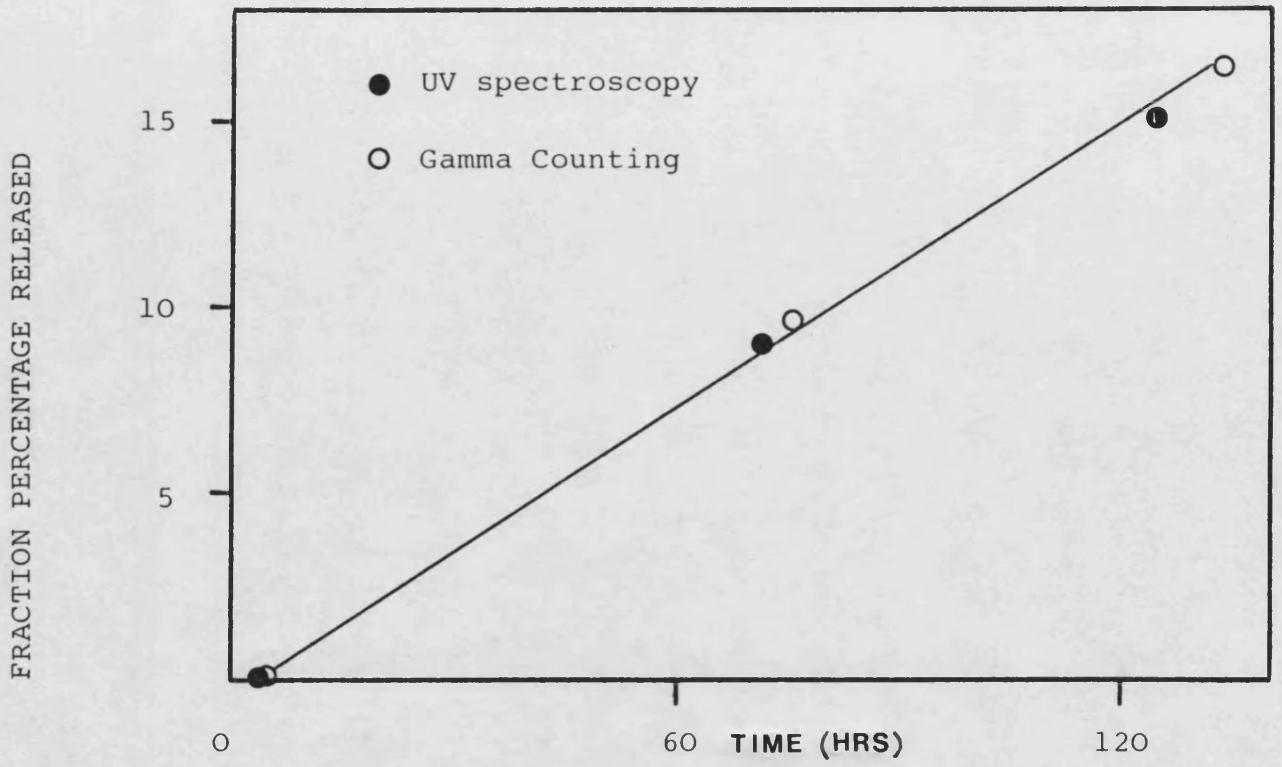



FIGURE 6.2 ELECTROPHORETIC INSULIN RELEASE THROUGH 30T 7C POLYACRYLAMIDE GEL AT A CONSTANT 15 VOLTS

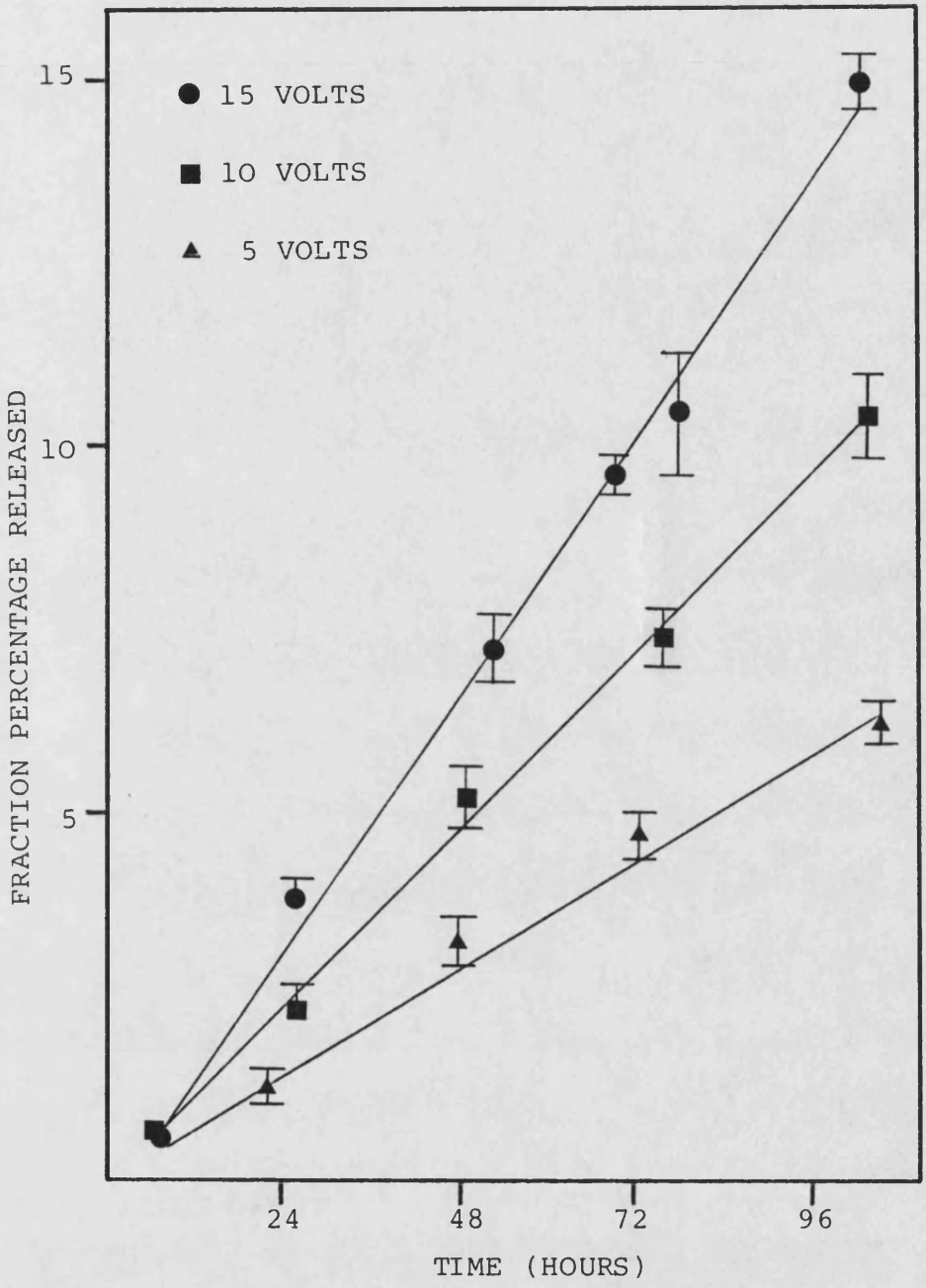



FIGURE 6.3 ELECTROPHORETIC RELEASE OF INSULIN THROUGH 30T 7C POLYACRYLAMIDE GEL VERSUS APPLIED VOLTAGE

remained undetected in the receptor compartment after more than 100 hours of electrophoresis indicating that 30T 7C polyacrylamide gel discs provided an adequate barrier (over a period of 4 days) to the permeation of insulin from the donor solution (as postulated in section 4.6).

Application of a constant 2.5 volts to the electrophoresis apparatus was found inadequate to produce a detectable insulin release rate; however zero-order electrophoretic drug release was obtained by application of constant voltage at 5,10 and 15 volts; linear correlation coefficients for the release profiles were 0.998 (5V), 0.992 (10V) and 0.998 (15V).

Insulin release rateswere calculated from the slopes of the release profiles in figure 6.3 and were found to increase with applied voltage. Insulin release rates (I.U. per day) were 8.1 (5V), 12.8 (10V) and 16.5 (15V) and a plot of insulin release rate versus applied voltage (figure 6.4) indicates an approximately linear relationship in the range 5 to 15V. The daily insulin requirement of diabetics is found to vary widely e.g. between 10 and 100 I.U. [193].

The linear relationship between applied voltage (5 to 15 volts) and insulin release rate is analogous to that found between applied voltage (10 to 275 volts) and insulin-F migration velocity in rod gel electrophoresis (section 5.2.1.). Thus the electric field generated within the rod gel and 2-compartment electrophoretic release apparatus is believed to be directly proportional to applied voltage. Loss of linearity below 10 volts in the relationship between applied voltage and insulin-F migration velocity in rod gel electrophoresis was observed (section 5.3.); however, this effect was not found at

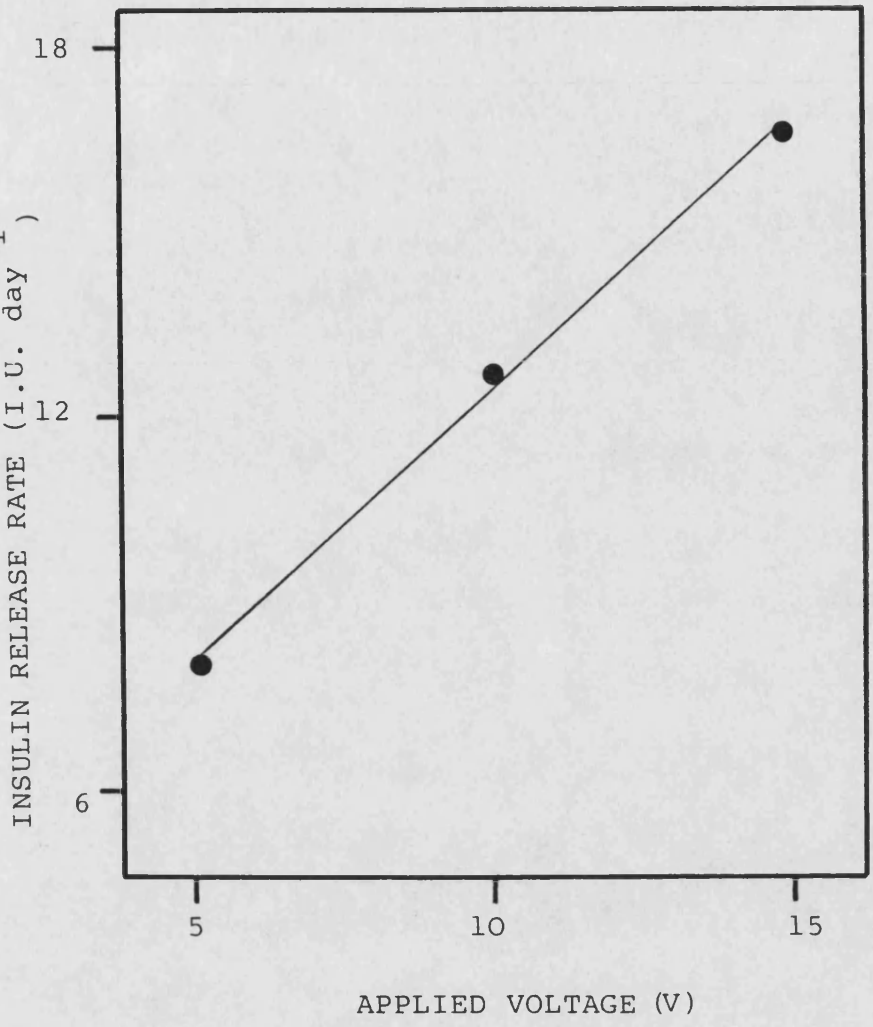



FIGURE 6.4 INSULIN RELEASE RATE VERSUS APPLIED VOLTAGE IN 2-COMPARTMENTAL ELECTROPHORETIC RELEASE THROUGH 30T 7C POLYACRYLAMIDE GEL DISCS

10V in 2-compartmental release studies because use of 30T 7C polyacrylamide gels reduced the effect of insulin diffusion previously observed in 5T 5C rod gels.

Figure 6.5 shows that current increased from 0.6 mA to 0.8 mA in the first 24 hours of electrophoretic release of insulin (at a constant 5V) and then decreased gradually to a value of 0.7 mA after approximately 72 hours. The initial increase in current is considered to be due to decreased electrical resistance of the polyacrylamide disc as the charge-carrying insulin molecules pass into the gel. The subsequent decrease in current is believed to result from a number of factors including buffer-ion migration and electrolytic effects (section 1.5.4.4.v). Similar profiles i.e. an initial increase followed by a decreasing and then more stable current were found at 10 and 15 volts.

(b) **Polyacrylamide gel concentration**

Increasing the total monomer concentration (T) of polyacrylamide gel discs in the range 20 to 30T was found to result in a decreased rate of electrophoretic insulin release (at zero-order) at a constant 5V (figure 6.6). This is considered to be due to the decrease in polyacrylamide gel porosity with increasing T which causes greater retardation of the insulin molecule.

Insulin release rates were determined from the slopes of drug release profiles and found to be 7.5 (30T gel), 9.0 (25T gel) and 10.9 (20T gel) I.U. per day. A plot of insulin release rate versus T indicates a linear relationship (figure 6.6) analogous to that previously found between insulin-F migration velocity and T (in the range 10 to 25%) in rod gel electrophoresis (section 5.3.).

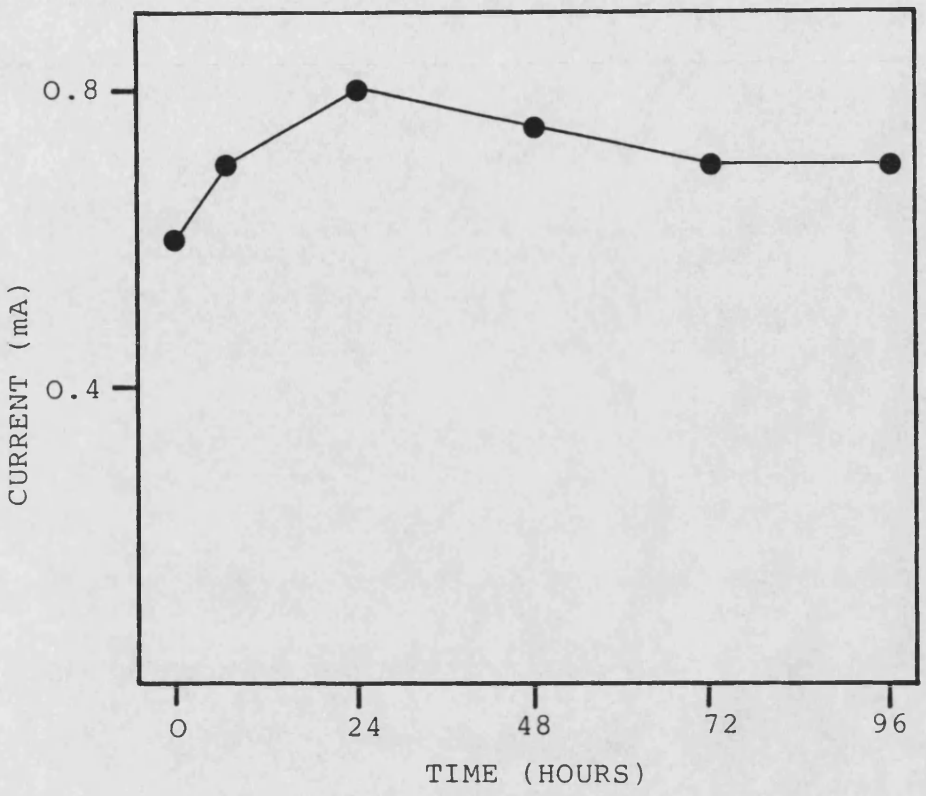



FIGURE 6.5 ELECTRIC CURRENT PROFILE DURING ELECTROPHORETIC INSULIN RELEASE AT A CONSTANT 5 VOLTS

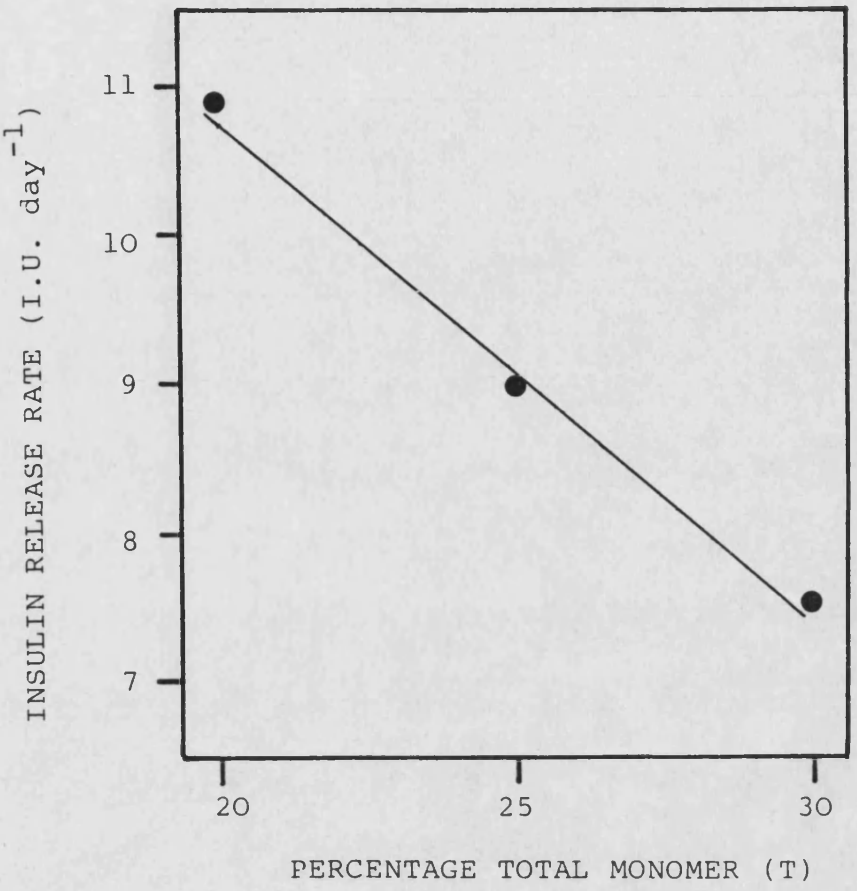



FIGURE 6.6 EFFECT OF PERCENTAGE TOTAL MONOMER (T) ON INSULIN RELEASE RATE THROUGH POLY-ACRYLAMIDE GEL (7C) AT A CONSTANT 5 VOLTS

The above results indicate that desired electrophoretic insulin release rates may be obtained by simple manipulation of applied voltage (field strength) and polyacrylamide gel formulation.

**(c) Alteration of electric field conditions**

**(I) Repeated switching on and off of electric field**

Figure 6.7 shows receptor insulin concentration during electrophoretic release employing the intermittent application of 5 volts; the release profile has 4 regions:

(1) After a lag time of approximately 4 hours, insulin is released at 8.3 I.U. $day^{-1}$ (i.e. at 5 volts).
(2) Drug release rate falls to zero at 24 hours (corresponding to the removal of the electric field at that point).
(3) Re-application of an electric field (5 volts) effects an insulin release rate of 8.4 I.U. $day^{-1}$.
(4) When the voltage is switched off, insulin release stops.

Interestingly (excepting the initial lag period), insulin release commences and ceases immediately on application and removal of the electric field - a highly desirable feature in terms of obtaining true controlled drug release.

**(II) Application of 5 and 10V alternately**

Figure 6.8 shows receptor insulin concentration during electrophoretic release using 5 and 10 volts, applied alternately; the release profile indicates 5 distinct phases:

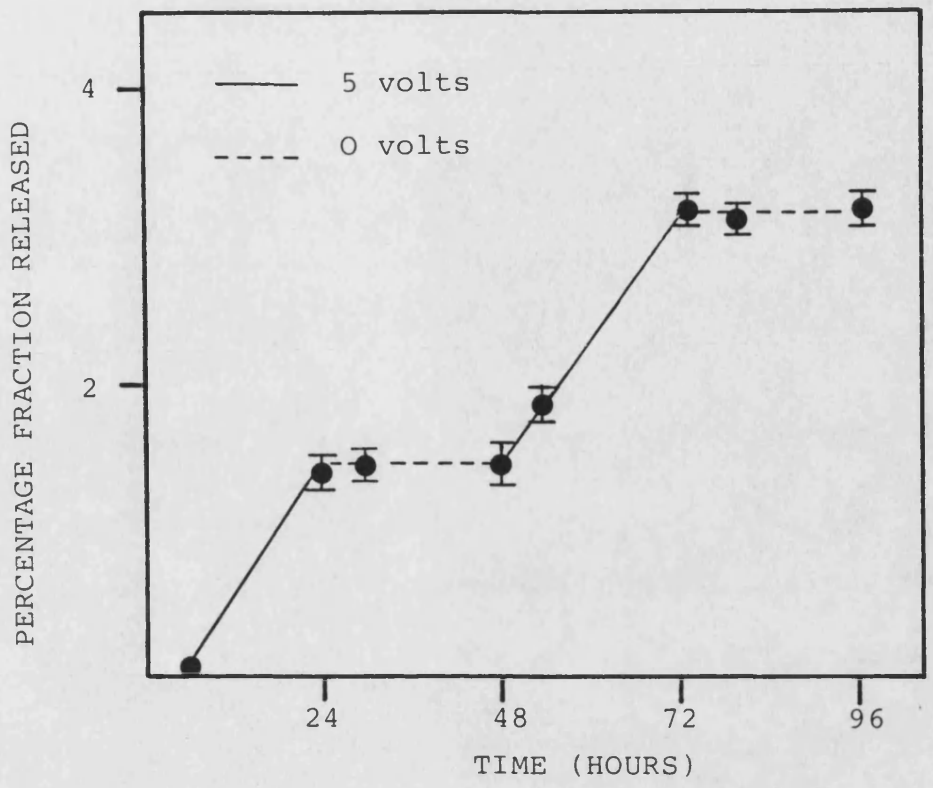



FIGURE 6.7 ELECTROPHORETIC RELEASE OF INSULIN THROUGH 30T 7C POLYACRYLAMIDE GEL AT AN ALTERNATING 5 AND ZERO VOLTS

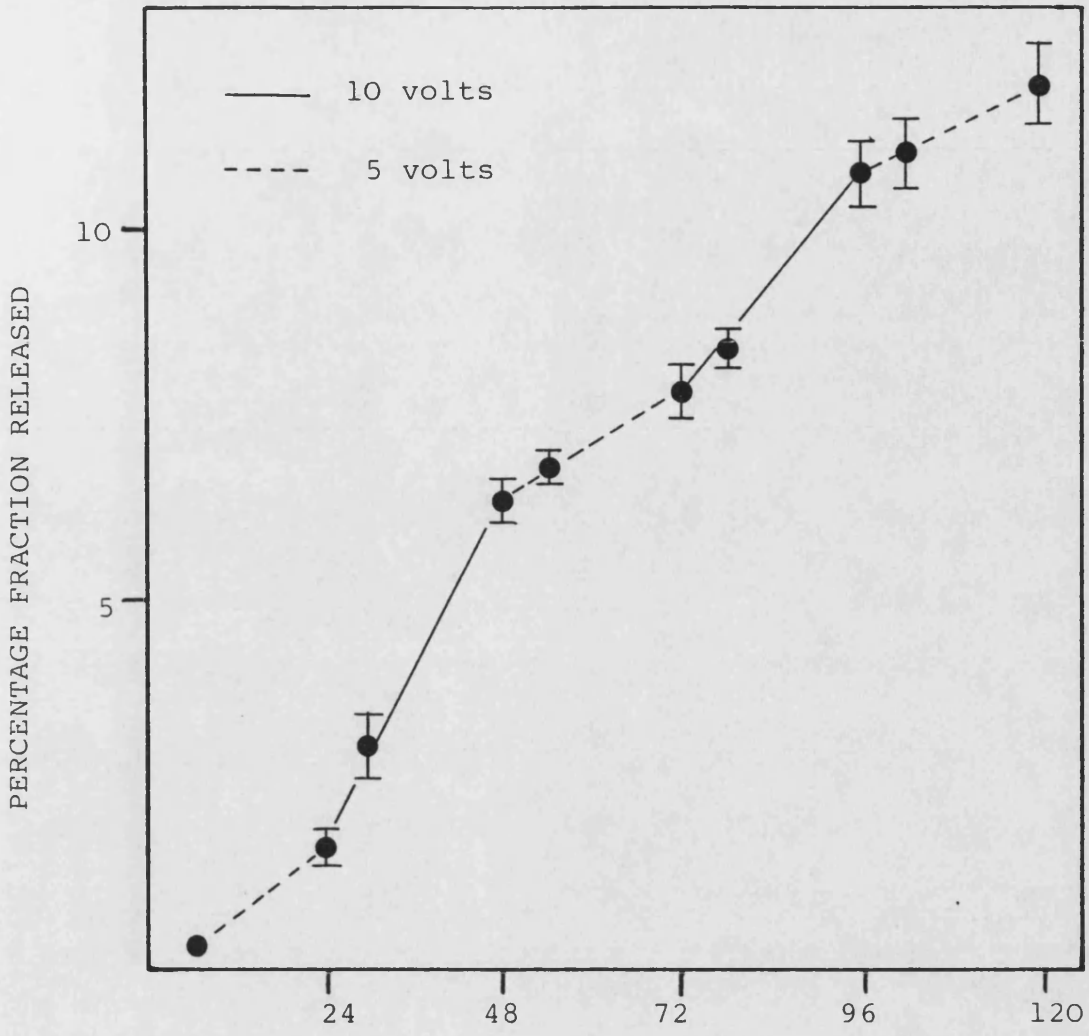



FIGURE 6.8 ELECTROPHORETIC RELEASE OF INSULIN THROUGH 30T 7C POLYACRYLAMIDE GEL AT AN ALTERNATING 5 AND 10 VOLTS

(1) An initial release rate of 8.0 I.U. $day^{-1}$ corresponding to an applied potential difference of 5 volts.

(2) Between 24 and 48 hours a constant 10 volts was applied and insulin release rate was found to rise to 23.4 I.U. $day^{-1}$.

(3) A release rate of 8.4 I.U. $day^{-1}$ was observed when 5 volts was applied once again between 48 and 72 hours.

(4) Re-application of 10 volts produced a release rate of 13.9 I.U. $day^{-1}$ (72 to 96 hours).

(5) Insulin was released at 5.5 I.U. $day^{-1}$ between 96 and 120 hours at a constant 5 volts.

Although based on only 3 data points, each phase indicates zero-order release. Insulin release rate during phase (2) was higher than expected on the basis of a 12.8 I.U. $day^{-1}$ rate previously obtained (figures 6.3 and 6.4) when a potential difference of 10 volts was also employed. A reduction in applied voltage from 10V to 5V at 48 hours caused a fall in insulin release rate to 8.4 I.U. $day^{-1}$. 10 volts (phase 4) and 5 volts (phase 5) produced release rates of 13.9 and 5.5 I.U. $day^{-1}$ respectively. These values were lower than obtained during earlier phases utilising the same voltage conditions; it is believed that donor drug depletion accounts for the apparent decrease in release rate. However, the marked increase in insulin release rate on going from phase 1 to phase 2 is difficult to explain. It is possible that insulin concentration changes within the gel, caused by the voltage changes described, are responsible. In addition, physical changes in polyacrylamide gel caused by the electric field changes or swelling cannot be ruled out. The effects of electrode polarization may have resulted in field strength

changes not detected by voltage and current measurements.

Since intermittent application of an electric field resulted in more reproducible release rates, it is considered that this type of electric field change would be most useful for obtaining controlled drug release. It is envisaged that release rate could be modulated by alteration of (D.C.) frequency of electric field application.

**(iv) Buffer electrolyte pH changes during electrophoretic insulin release**

Buffer pH's of donor and receptor compartments were found to change during the course of electrophoretic release experiments. Donor pH increased by between 0.5 and 1.0 pH unit while that of the receptor decreased by the same amount. The above pH changes can be explained on the basis of electrolysis effects (section 1.5.4.4.v) and no attempts were made to improve buffering capacities. However, the effect of ionic strength of McIlvane's citrate-phosphate buffer (for electrophoretic release studies) on buffering capability was studied (section 6.4.1.).

### 6.5.2. Electrophoretic release of hyoscine methyl chloride

**(i) Verification of electrophoretic hyoscine methyl chloride release**

The radiolabelled receptor solution sample produced an identical peak to that obtained after HPLC of a $1.4 \times 10^{-5}$ mol $dm^{-3}$ standard solution of hyoscine methyl chloride (figure 2.9). This suggested that degradation of hyoscine methyl chloride probably did not occur during release experiments and that the liquid scintillation assay of

this drug was satisfactory.

### (ii) Factors affecting electrophoretic release of hyoscine methyl chloride

#### (a) Applied potential difference

Figure 6.9 shows hyoscine methyl chloride electrophoretic release rates using various applied voltages (field strengths).

Drug remained undetected in the receptor after 72 hours at zero volts. This confirms the postulation made in section 4.6. that 40T 7C polyacrylamide gel discs of 10 mm thickness would limit the permeation of hyoscine methyl chloride to less than 0.7% in 4 days.

Application of between 5 and 20 volts produced zero-order drug release; linear correlation coefficients of hyoscine methyl chloride release profiles were 0.993 (5 volts), 0.999 (10 volts), 0.998 (15 volts) and 0.999 (20 volts). Hyoscine methyl chloride release rate increased with applied voltage; shortest lag times for release (ranging from approximately 0.5 to 4 hours) were associated with the highest release rates. A hyoscine methyl chloride release rate equivalent to $3.0 \times 10^{-6}$ mol $day^{-1}$ was found using 40T 7C gels at a constant 10 volts.

Under similar electrophoresis conditions, a greater fraction of hyoscine methyl chloride was released compared with percentage fraction insulin released (section 6.5.1.). This was probably due to the smaller molecular size of hyoscine methyl chloride (section 2.1.1.), resulting in faster electrophoretic migration. In the absence of information concerning porcine insulin surface charge under the conditions used in the present study, it is difficult to discuss the relative influence of surface charge on the

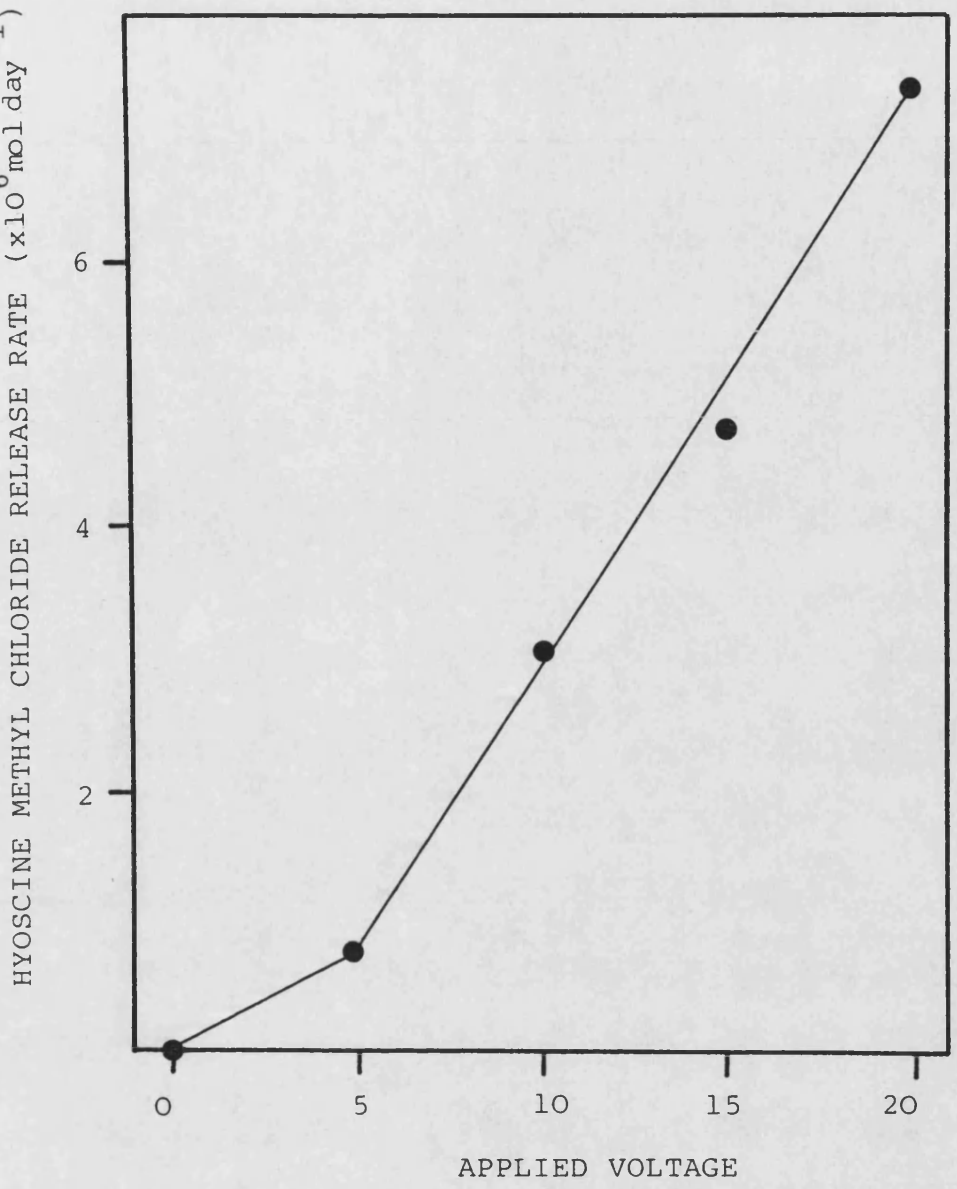



FIGURE 6.9 EFFECT OF APPLIED VOLTAGE ON ELECTROPHORETIC RELEASE OF HYOSCINE METHYL CHLORIDE THROUGH 4OT 7C POLYACRYLAMIDE GEL

electrophoretic release rates of hyoscine methyl chloride and insulin.

A plot (figure 6.9) of applied voltage versus hyoscine methyl chloride release rate (mol $day^{-1}$) indicates a linear relationship between 5 and 20 volts; the curve loses linearity below 5 volts. Extrapolation of the straight line portion to the voltage axis suggests that approximately 4 volts must be applied to the electrophoretic release apparatus before drug release is effected. This may indicate a degree of drug "immobilization" by the polyacrylamide network which is overcome by application of an appropriate electric field produced by approximately 4 volts potential difference.

(b) **Polyacrylamide gel concentration**

Figure 6.10 shows the effect of increasing T on hyoscine methyl chloride release rate (mol $day^{-1}$) at a constant 10 volts. As previously found during electrophoretic insulin release (section 6.5.1.), a linear relationship was observed suggesting increased retardation of drug molecules by the polyacrylamide gel network as T is increased.

(c) **Alteration of electric field conditions during delivery**

Figure 6.11 shows that at a constant 10 volts, and after a lag time of approximately 5 hours, hyoscine methyl chloride was released through a 40T 7C polyacrylamide gel disc at a constant rate ($3.3. \times 10^{-6}$ mol $day^{-1}$). Removal of the electric field after 24 hours caused drug release to cease immediately but was restored to

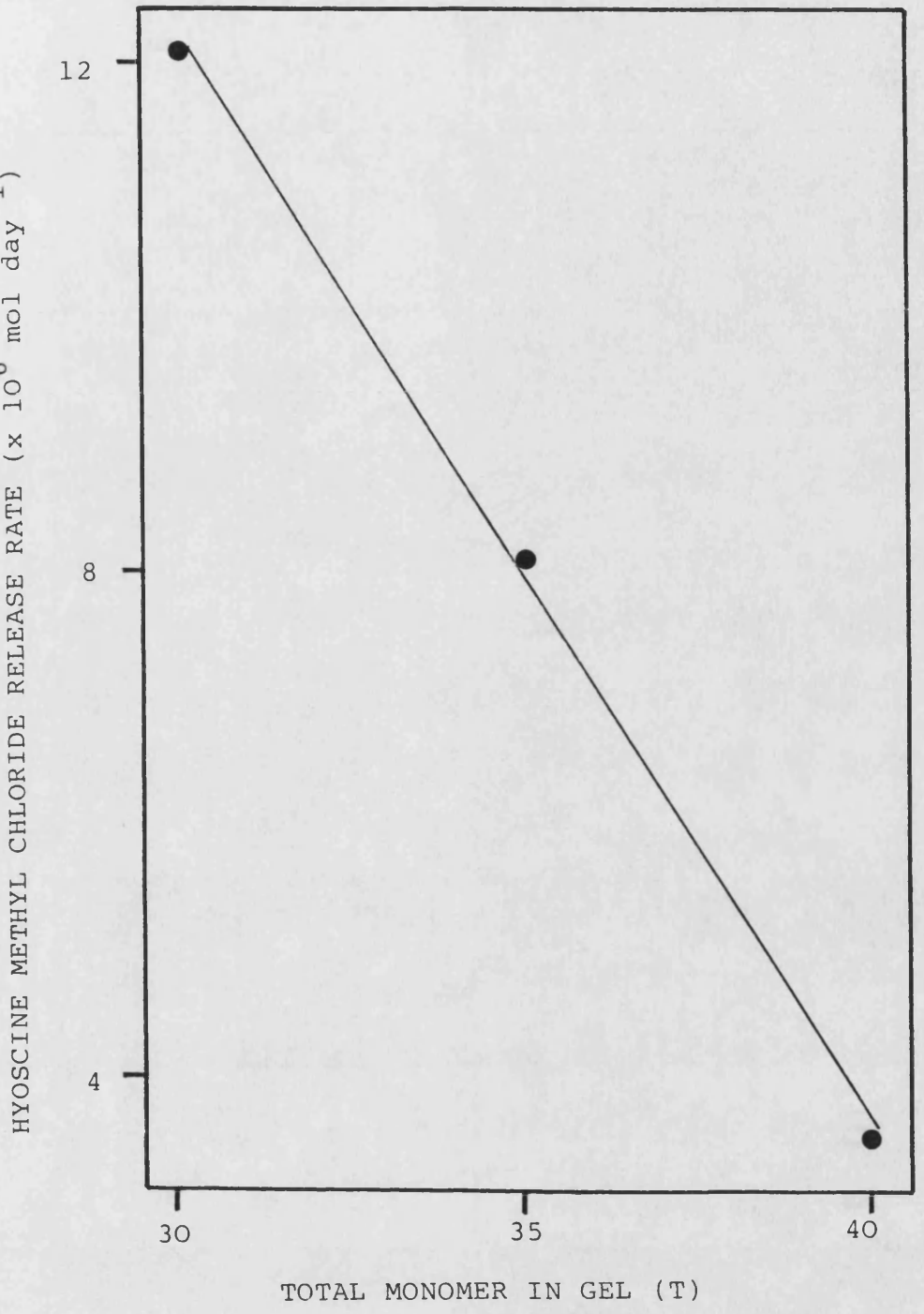



FIGURE 6.10 EFFECT OF TOTAL MONOMER CONCENTRATION (T) OF POLYACRYLAMIDE GEL ON ELECTROPHORETIC RELEASE RATE OF HYOSCINE METHYL CHLORIDE (AT 10 VOLTS)

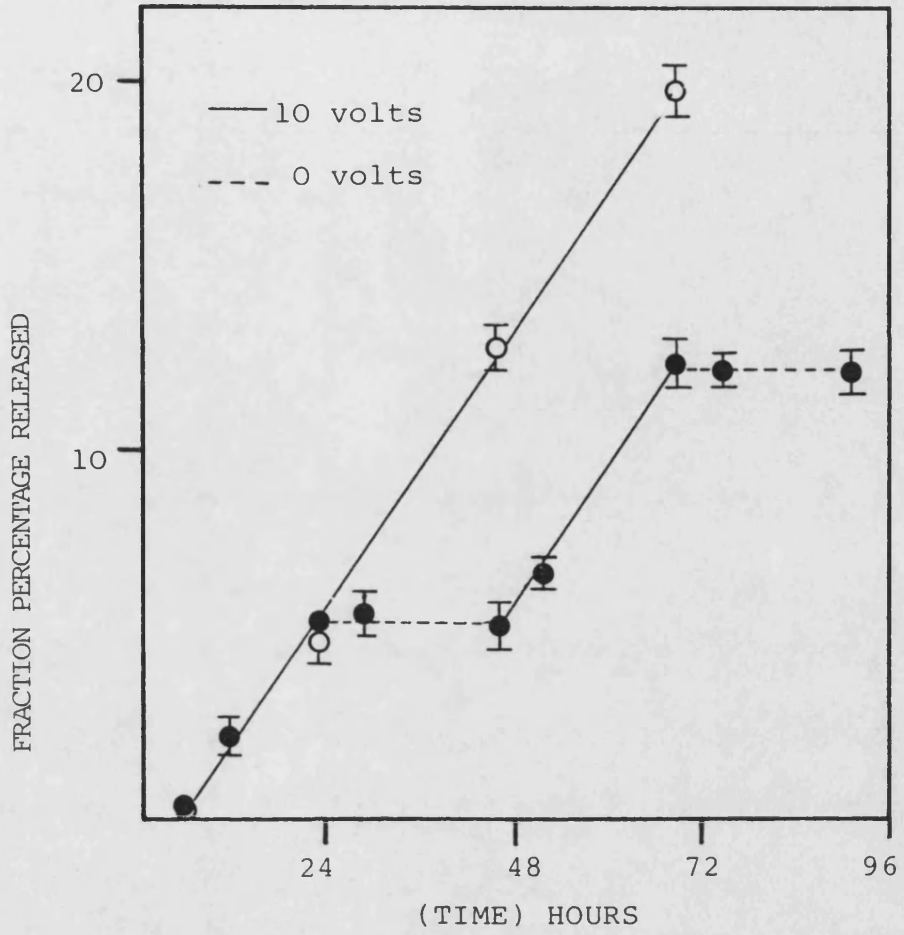



FIGURE 6.11 ELECTROPHORETIC RELEASE OF HYOSCINE METHYL CHLORIDE AT 10 VOLTS AND ALTERNATING 10/0 VOLTS THROUGH 4OT 7C POLY-ACRYLAMIDE GEL

the above rate on re-applying the field at 48 hours. A second switching off of applied voltage at 72 hours again resulted in immediate cessation of drug release.

The above results indicate that gross morphological changes in polyacrylamide gel structure probably do not occur as a result of electrophoresis. The reproducibility of release rate during the 2 periods of electrophoretic drug release suggest that as well as macromolecules such as insulin (section 6.5.1.), drugs of low molecular weight can also be electrophoretically released in a highly controllable manner through polyacrylamide gel.

(d) **Temperature**

A plot of current (at a constant 10 volts) versus temperature of electrophoretic release shows a linear relationship (figure 6.12). Figure 6.13 indicates an approximately linear relationship between temperature and hyoscine methyl chloride release rate. Both curves are analogous to those obtained during rod gel electrophoresis when the migration velocity of insulin-F was studied (section 5.3). It is believed that the causes of increased insulin-F migration velocity with rising temperature discussed above in section 5.3. are also responsible for the increase in hyoscine methyl chloride release rate with temperature.

(e) **Polyacrylamide thickness**

In figure 6.14 the effect of increasing polyacrylamide gel thickness on the electrophoretic release of hyoscine methyl chloride at a constant 10 volts is shown. As expected, lag time decreased

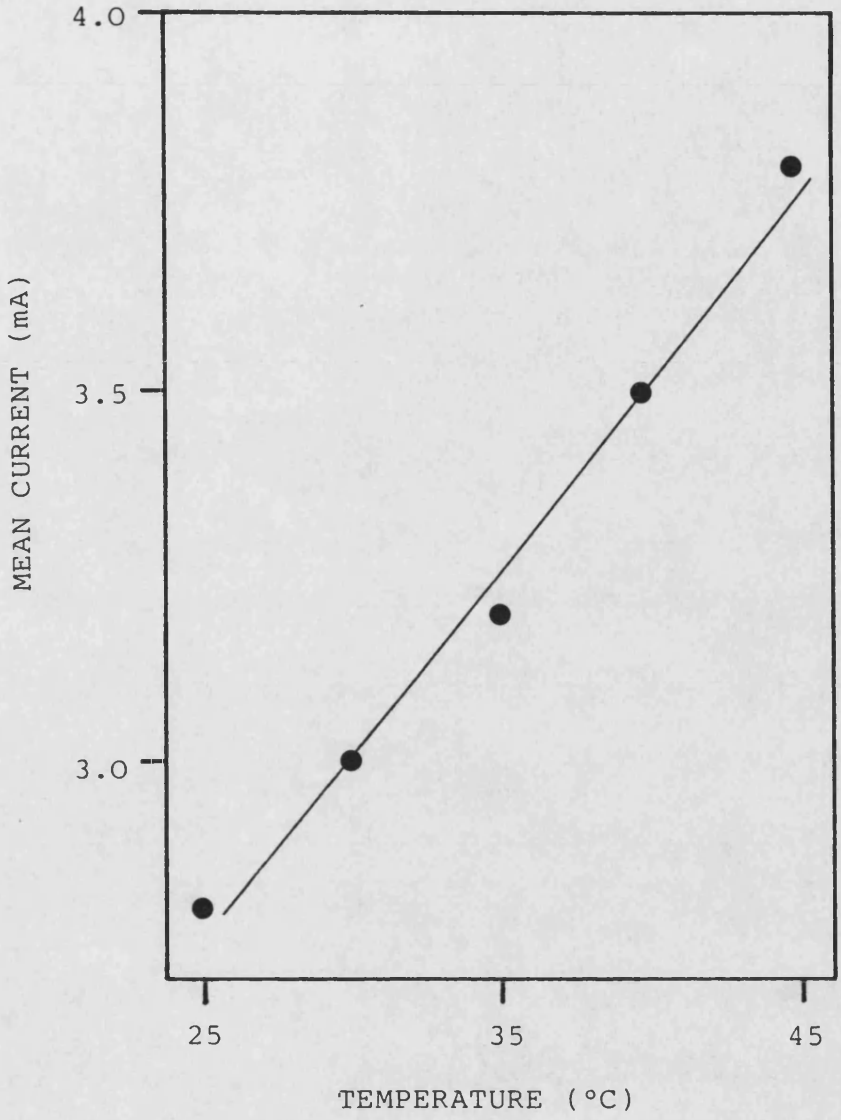



FIGURE 6.12 EFFECT OF TEMPERATURE ON CURRENT DURING ELECTROPHORETIC RELEASE OF HYOSCINE METHYL CHLORIDE AT A CONSTANT 10 VOLTS

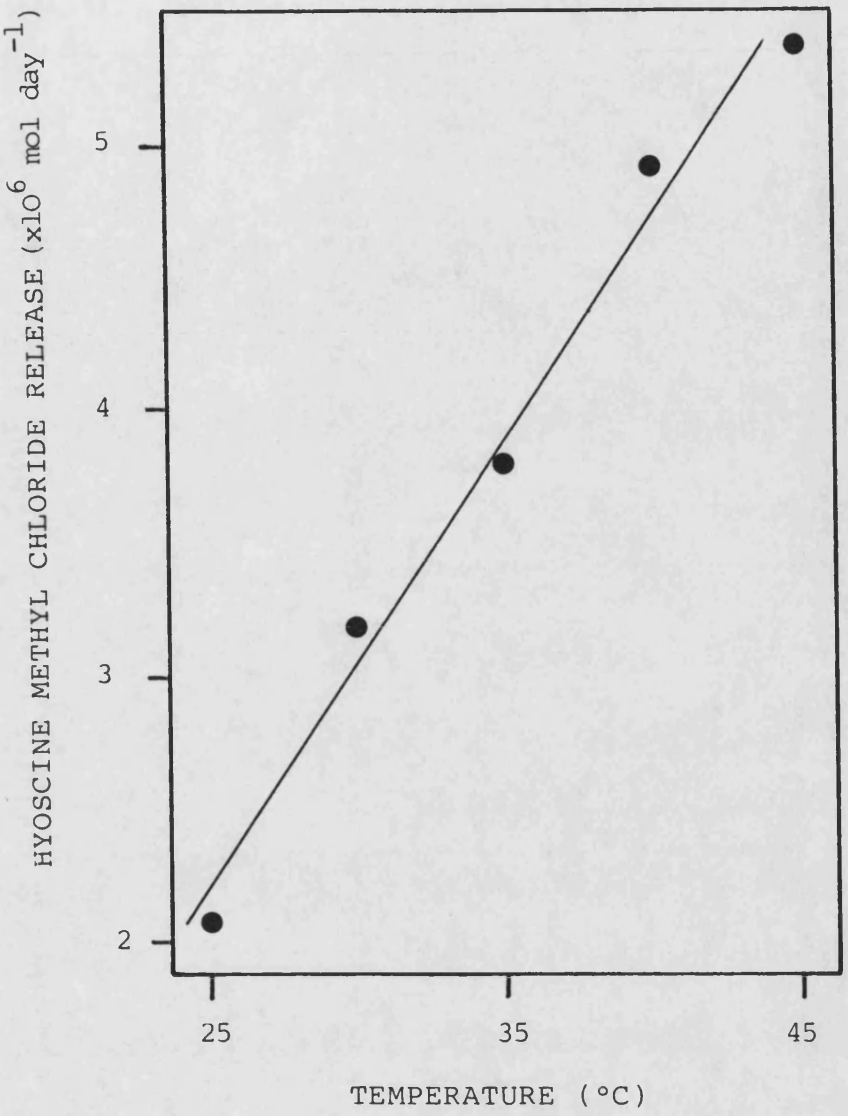



FIGURE 6.13 EFFECT OF TEMPERATURE ON ELECTROPHORETIC HYOSCINE METHYL CHLORIDE RELEASE THROUGH 4OT 7C POLYACRYL-AMIDE GEL AT A CONSTANT 1O VOLTS

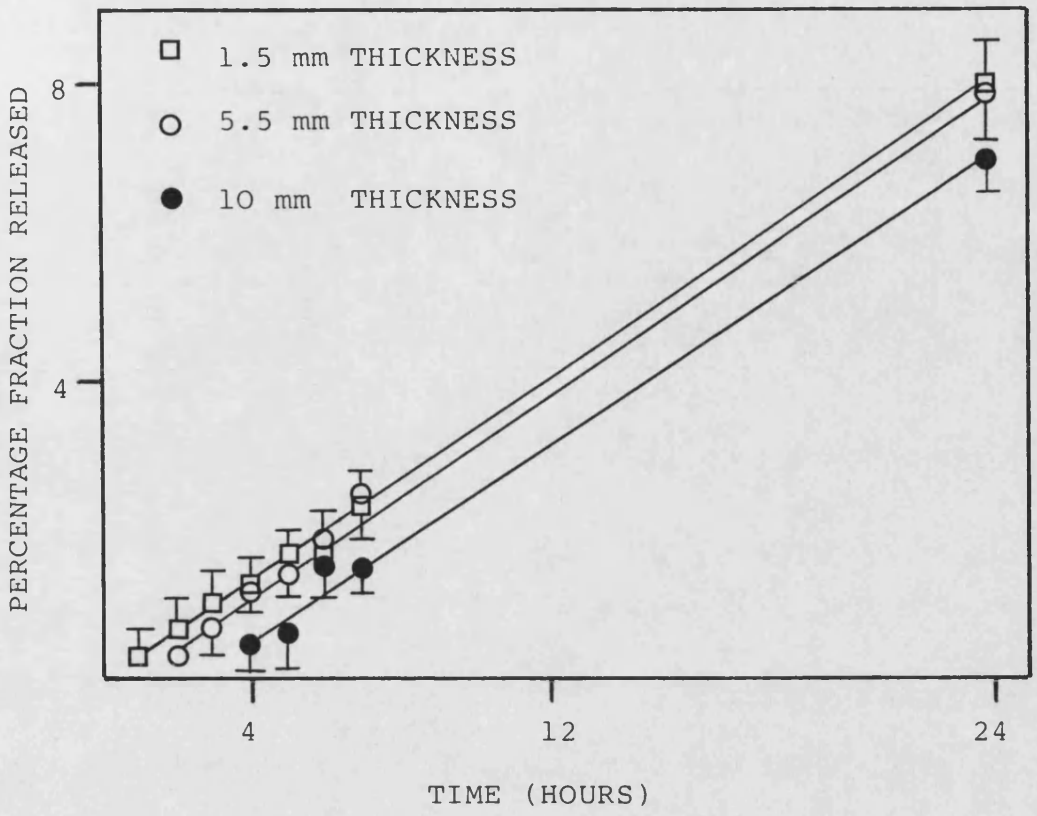



FIGURE 6.14 EFFECT OF POLYACRYLAMIDE GEL THICKNESS ON ELECTROPHORETIC RELEASE OF HYOSCINE METHYL CHLORIDE AT A CONSTANT 10 VOLTS

with decreasing gel thickness, since drug migration distance was reduced. From figure 6.14 drug release rate appears to be unaffected by gel thickness. However, electrophoretic release over longer periods than 24 hours would be expected to produce higher "apparent" release rates as gel thickness is reduced because of the increased contribution to release by drug permeation (sections 4.4.2. and 4.6.).

(f) **Donor drug concentration**

Figure 6.15 shows that a complex relationship exists between hyoscine methyl chloride release rate and change in donor drug concentration. The release rate (as a percentage fraction of donor concentration) from $2.82 \times 10^{-4}$ and $2.82 \times 10^{-3}$ mol $dm^{-3}$ donor solutions was approximately equal, while from $2.82 \times 10^{-5}$ mol $dm^{-3}$ donor solution, a slower release rate was found.

The current flowing through the apparatus during the course of delivery was found to increase with increasing donor drug concentration due to the greater number of charge-carriers present. Mean currents were 2.0 mA, 3.0 mA and 3.5 mA for the $2.82 \times 10^{-5}$, $2.82 \times 10^{-4}$ and $2.82 \times 10^{-3}$ mol $dm^{-3}$ donor drug solutions respectively. It is believed that the lower release rate from the $2.82 \times 10^{-5}$ mol $dm^{-3}$ donor drug solution was due to the low current flowing during release and the expected differentiation between drug release rates from the more concentrated donor solutions on the basis of current flowing was not clear in this study.

Data from the highest donor drug concentration ($2.82 \times 10^{-2}$ mol $dm^{-3}$) is omitted because it was found to be highly irreproducible, in addition, a green colouration developed in the donor compartment, probably due to the evolution of chlorine by electrolysis. This may

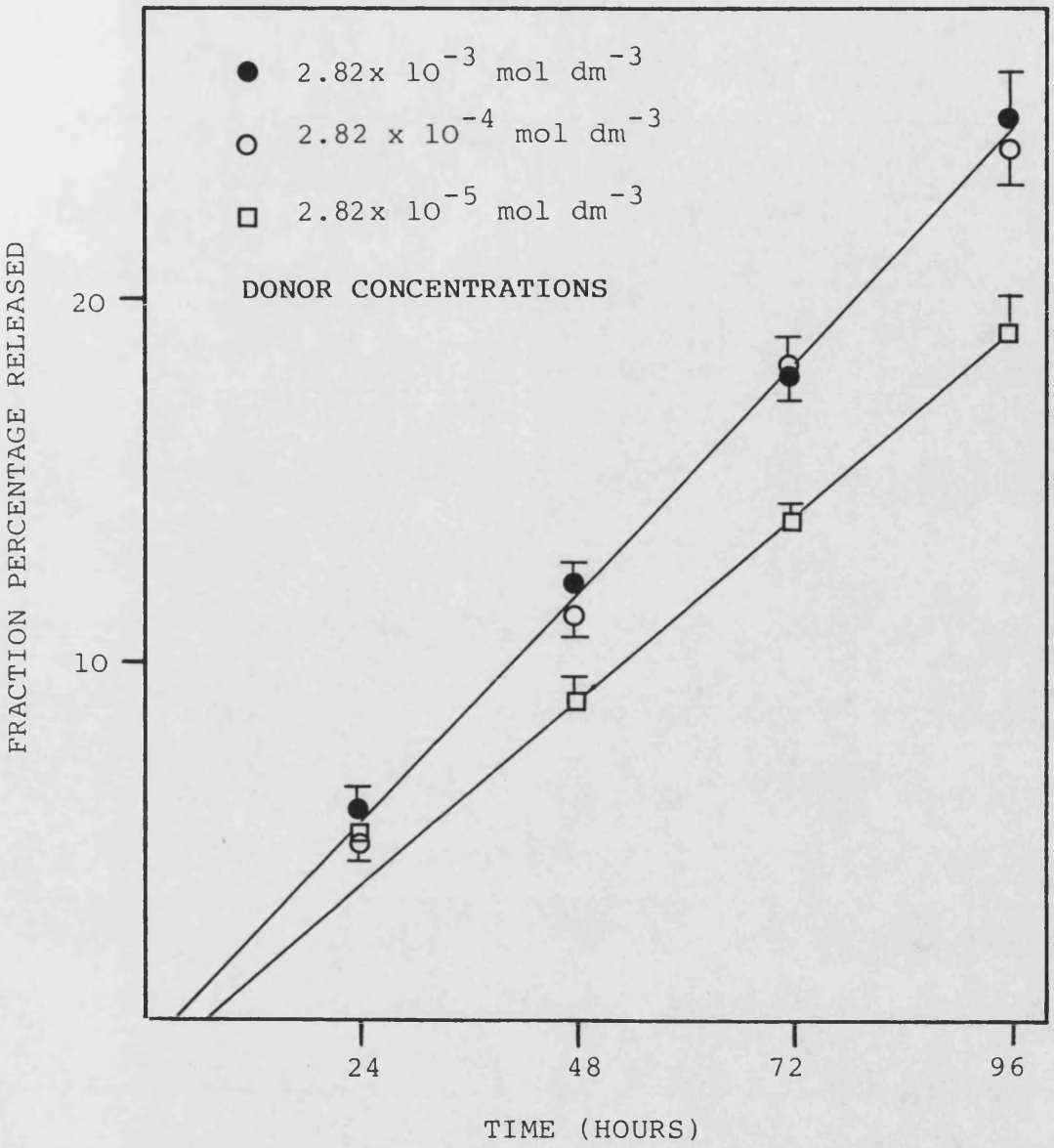



FIGURE 6.15 EFFECT OF DONOR DRUG CONCENTRATION ON ELECTROPHORETIC HYOSCINE METHYL CHLORIDE RELEASE THROUGH 4OT 7C POLYACRYLAMIDE GEL AT A CONSTANT 1O VOLTS

have been reponsible for the errors detected in electrophoretic delivery. No discoloration was observed in the more dilute donor drug solutions during electrophoresis.

The relationship between hyoscine methyl chloride release rate and donor drug concentration is not clear. It is believed that if the above study was repeated using donor solutions of equal ionic strength (and thus equivalent electric currents during electrophoresis), more useful results would be obtained.

(g) **Duration of electrophoretic release**

Figure 6.16 shows that hyoscine methyl chloride was electrophoretically released at a constant rate ($3.5 \times 10^{-6}$ mol $day^{-1}$) up to approximately 150 hours, release rate then gradually falls. Interestingly, electric current (at 10 volts) in the release apparatus increased gradually from the start of delivery (2.2 mA) and reached a constant value of 4.8 mA after approximately 150 hours.

2-compartmental permeation experiments using membranes e.g. skin diffusion studies are generally discontinued once 10% donor depletion has occurred since permeation rate decreases as donor concentration falls [215]. Similarly, electrophoretic release rate would be expected to decrease as depletion of the donor occurs. It is believed that the steadily increasing current maintained a constant release rate of hyoscine methyl chloride; when the current became more stable, drug release rate fell. The causes of electric current increase changes during electrophoretic drug release are discussed below.

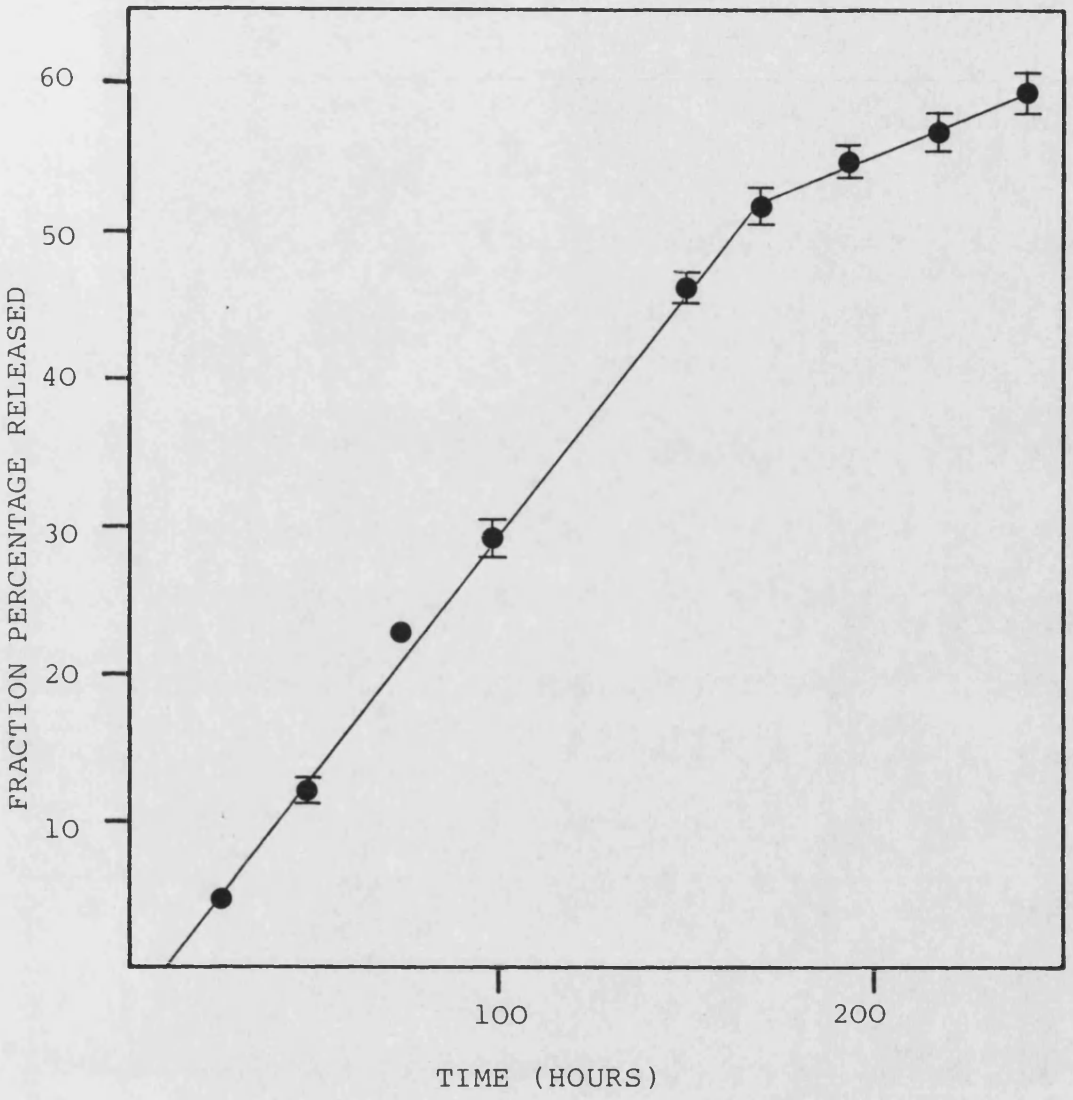



FIGURE 6.16 ELECTROPHORETIC RELEASE OF HYOSCINE METHYL CHLORIDE THROUGH 40T 7C POLY-ACRYLAMIDE GEL AT A CONSTANT 10 VOLTS

### (iii) Changes in electric current and electrolyte pH during hyoscine methyl chloride release

Electric current was found to increase during the course of hyoscine methyl chloride release. For example, at a constant 10 volts, current typically increased from 2 mA to 4 mA over 72 hours thus giving a mean current for the run of 3 mA. This increase was believed to be due to (a) a reduction in polyacrylamide gel electrical resistance as drug passes into the gel disc and (b) electrochemical reactions occurring at the electrodes.

The pH of the donor solution was found to decrease by less than one pH unit after 72 hours of electrophoresis at 10 volts while that of the receptor solution increased by approximately the same amount. Electrochemical reactions and ionic migration are believed to be mainly responsible for the observed pH changes which were considered to be within acceptable limits.

## 6.5.3. Electrophoretic release of hyoscine hydrobromide and propranolol hydrochloride

Electrophoretic release profiles for hyoscine hydrobromide and propranolol hydrochloride are shown in figures 6.17 and 6.18 respectively.

Mean delivery currents were found to be $3.1 \pm 0.2$ mA (5 volts) and $4.5 \pm 1.6$ mA (10 volts) during hyoscine hydrobromide release and $4.2 \pm 0.6$ mA (5 volts) and $8.5 \pm 1.2$ mA (10 volts) during the release of propranolol. Current was observed to gradually increase during the course of electrophoretic delivery.

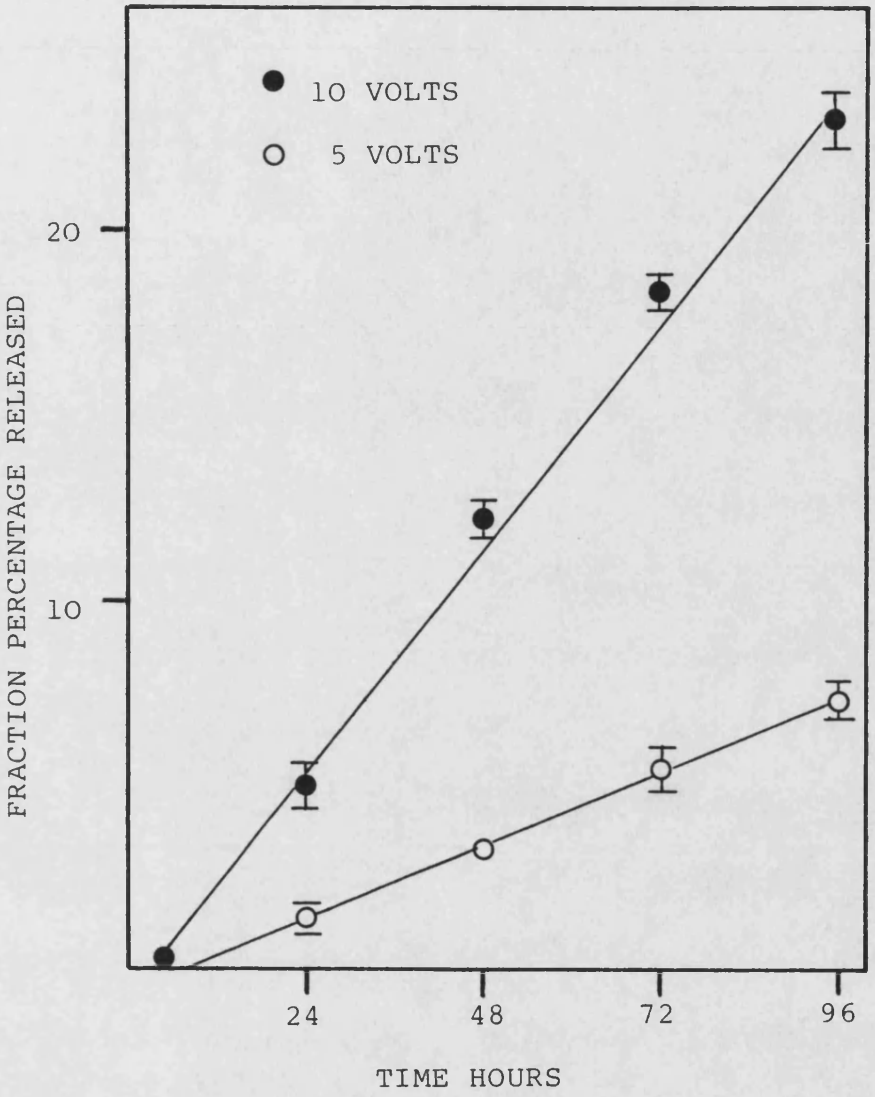



FIGURE 6.17 ELECTROPHORETIC RELEASE OF HYOSCINE HYDROBROMIDE THROUGH 4OT 7C POLY-ACRYLAMIDE GEL AT 5 AND 1O VOLTS

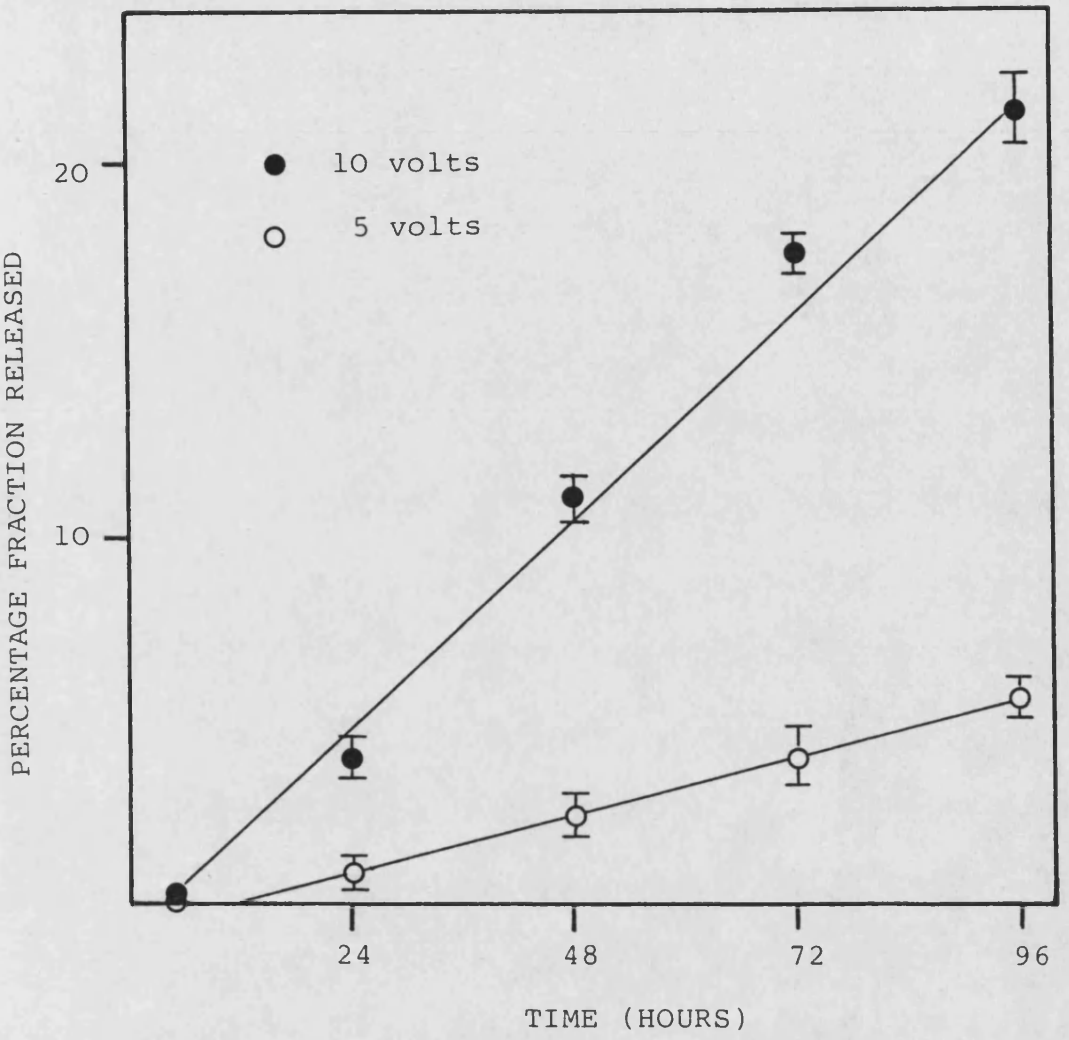



FIGURE 6.18 ELECTROPHORETIC RELEASE OF PROPRANOLOL HYDROCHLORIDE THROUGH 4OT 7C POLYACRYL-AMIDE GEL AT 5 AND 1O VOLTS

Hyoscine hydrobromide was found to be released at a constant rate by applying 5 volts ($0.8 \times 10^{-6}$ mol $day^{-1}$) or 10 volts ($2.4 \times 10^{-6}$ mol $day^{-1}$) for up to 96 hours; linear correlation coefficients were 0.990 (5 volts) and 0.988 (10 volts). A commercial system [51,52] delivers hyoscine hydrobromide to the skin at a rate of approximately $0.4 \times 10^{-6}$ mol $day^{-1}$. It is believed that a similar rate could be achieved using electrophoretically controlled hyoscine hydrobromide release by (i) decreasing donor drug concentration (ii) reduction of the applied voltage to below 5 volts and (iii) by manipulation of polyacrylamide gel formulation and dimensions.

Propranolol hydrochloride was released (at zero-order) at $3.3 \times 10^{-5}$ mol $day^{-1}$ (10 volts) and at $0.9 \times 10^{-5}$ mol $day^{-1}$ (5 volts); linear correlation coefficients were 0.999 (5 volts) and 0.995 (10 volts). Daily propranolol hydrochloride dose (by slow I.V. injection) is between $0.3 \times 10^{-6}$ mol and $3.4 \times 10^{-5}$ mol [193].

The above results indicate that both propranolol hydrochloride and hyoscine hydrochloride may be released at constant rates electrophoretically; the in vitro delivery of the latter to human skin was investigated (section 7).

Under similar electrophoretic conditions propranolol appears to be released at a greater rate than hyoscine hydrobromide, this was because a higher donor concentration of the former was initially present - fractions released were similar (figures 6.17 and 6.18).

# 7. *IN VITRO* ELECTROPHORETIC DELIVERY OF HYOSCINE HYDROBROMIDE TO HUMAN SKIN USING A 3-COMPARTMENT MODEL

## 7. *In vitro* electrophoretic delivery of hyoscine hydrobromide to human skin

Results obtained from 2-compartmental electrophoretic release studies (section 6.5) indicated that electrophoresis was potentially useful as an energy source for producing true controlled drug delivery. The *in vitro* controlled delivery of hyoscine hydrobromide to human skin was therefore investigated.

Drugs exhibiting transdermal penetration are usually most permeable in the unionized form (section 1.2.4.2.). However, ionized drug is required for electrophoresis; thus a 3-compartmental model utilising a multiphasic buffer system was proposed.

### 7.1. 3-Compartmental *in vitro* model for delivery of hyoscine hydrobromide to human skin

The delivery apparatus was constructed from 6 mm thickness perspex and is represented schematically in figure 7.1. Three chambers were clamped together in series, each sealed with a lid which was secured using brass bolts. Chambers 1 (drug reservoir) and 3 (receptor) were of equal capacity (150 $cm^3$) whilst compartment 2 had a lower capacity of 20 $cm^3$. PTFE stirring paddles rotating at 240 r.p.m. ensured solution mixing within each compartment. A section of human skin (section 2.1.4.) was held between compartments 2 and 3 with the stratum corneum surface exposed in the former. A rubber 'O' ring provided effective sealing and resulted in a circular skin permeation area of approximately 4 $cm^2$. A polyacrylamide gel disc of 10 mm thickness was held between chambers 1 and 2.

Compartment 2 contained hyoscine hydrobromide (pKa 7.35) solution, buffered at pH 9.7 to ensure the predominance of drug in

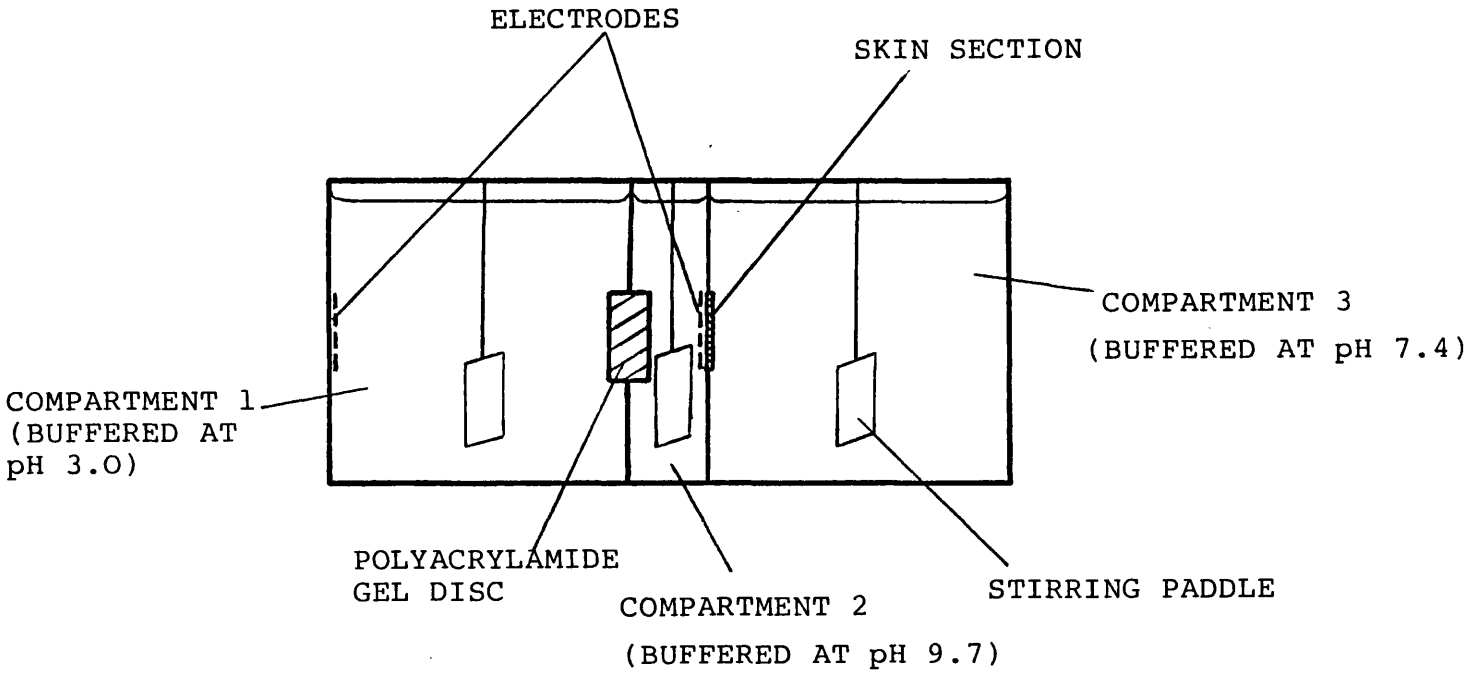



FIGURE 7.1 3-COMPARTMENTAL APPARATUS FOR IN VITRO ELECTROPHORETIC DRUG DELIVERY TO HUMAN SKIN

the unionized form thereby maximising its permeation through the skin section and into the receptor compartment which was buffered at pH 7.4.

Chamber 1 formed a hyoscine hydrobromide reservoir and was buffered at pH 3.0 to ensure complete ionization of the drug. A circular platinized (section 6.1.) electrode was embedded in the wall of the reservoir chamber and a second, circumjacent to the skin membrane in compartment 2.

It was intended that as drug concentration in compartment 2 became depleted through skin permeation, that hyoscine hydrobromide should be electrophoretically released from the reservoir at a rate equal to the transdermal flux. Thus drug concentration in compartment 2 would remain constant and provided that adequate sink conditions were maintained in the receptor, drug flux would be sustained at a constant level.

On the basis of results obtained from hyoscine methyl chloride electrophoretic release studies (section 6.5.2.) it was postulated that desired hyoscine hydrobromide delivery rates could be achieved by manipulation of donor drug solution concentration and electrophoretic voltage.

Initially, however, the effect of donor drug concentration and pH upon the transdermal permeation of hyoscine hydrobromide, the effectiveness of the proposed buffering system and drug stability at the proposed pH's were investigated in order to select appropriate conditions for the 3-compartmental study.

### 7.1.1. *In vitro* permeation of hyoscine hydrobromide through human skin

#### 7.1.1.1. Apparatus for skin permeation studies

An infinite dose technique (section 1.2.4.3.) was employed using perspex permeation cells (figure 7.2.) similar to those employed by Patel and Foss [59]. Epidermis was clamped between the 2 halves (each of 7 ml capacity) of the cell, a rubber 'O' ring on the stratum corneum side of the membrane ensured effective sealing; exposed skin area in each compartment was 4.9 $cm^2$. Samples were withdrawn through sampling ports otherwise sealed with PTFE coated stoppers.

#### 7.1.1.2 Preparation of epidermal sections for permeation experiments

Only relatively non-hairy abdominal cadaver skin samples were used from which "full thickness skin" membranes composed of stratum corneum, epidermis and some dermis were prepared according to a method described by Walters [58].

Skin samples were pinned, stratum corneum side down, and excess fat trimmed away leaving strips of uniform thickness. Specimens were then placed into distilled water at 60°C for precisely 2 minutes prior to being pinned down once more with the stratum corneum side facing upwards. The epidermis was cut across the membrane strip and carefully separated from the lower layers of skin using forceps. Full thickness skin membrane was then floated in distilled water onto a sheet of aluminium foil and left to dry for several hours before storage in sealed polythene bags at -5°C. Skin samples were used within one month. Before location into permeation

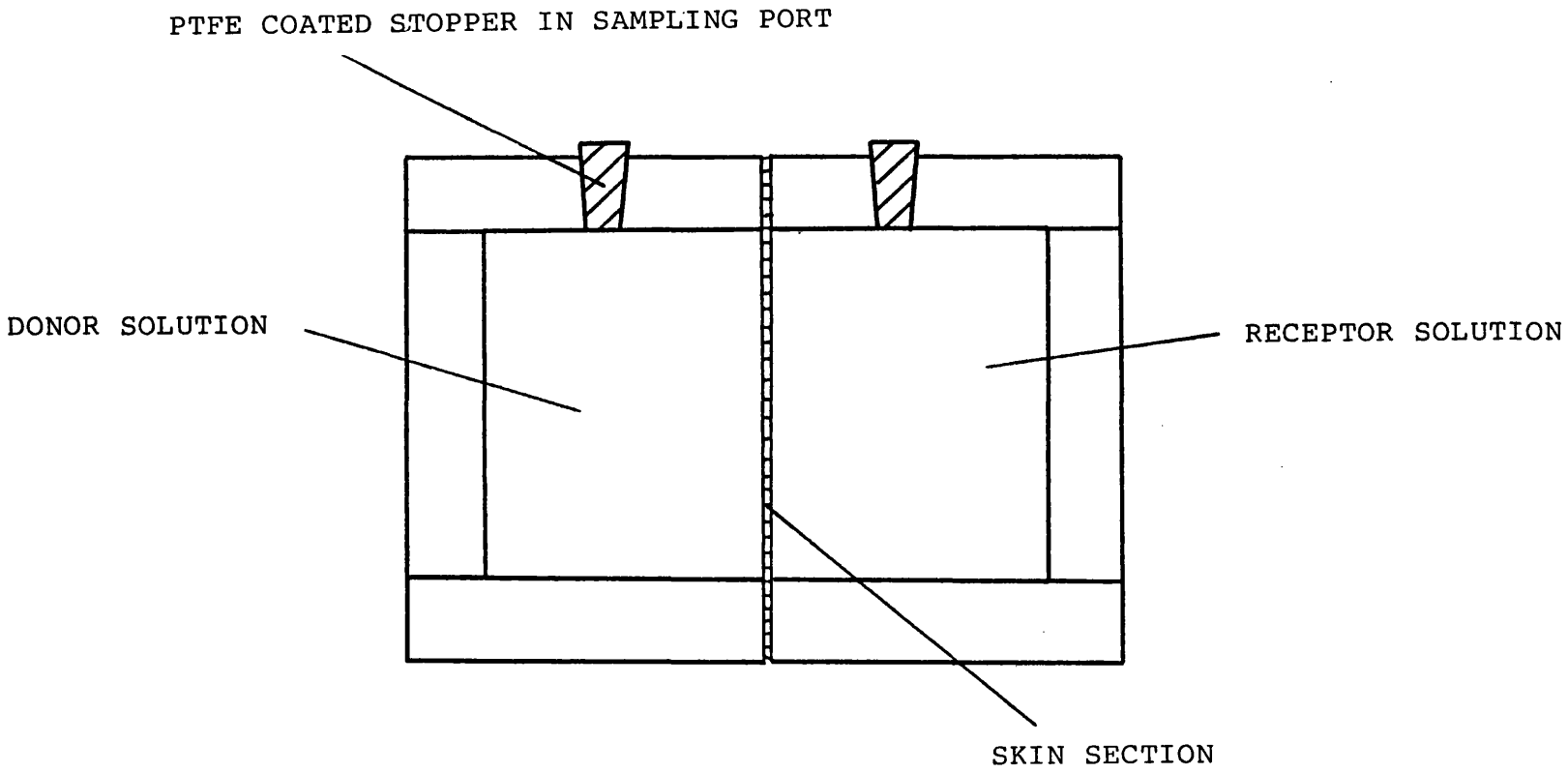



FIGURE 7.2 2-COMPARTMENT APPARATUS FOR IN VITRO SKIN PERMEATION STUDIES

cells, epidermal membranes were thawed out by placing them in distilled water for several hours; after this time, they could be separated from the aluminium foil sheets with ease and without damage.

### 7.1.1.3. General method for skin permeation studies

The permeation apparatus and donor and receptor solution were equilibrated to 30°C in a waterbath. 7 $cm^3$ of each solution was introduced into the appropriate chamber of the permeation cell simultaneously by means of glass pipettes so as to avoid undue distention of the epidermis. 1 x $10^{-3}$M phosphate buffer, pH 7.4, was employed in the receptor compartment, throughout the study; pH and simple electrolyte gradients across skin have been shown not to significantly affect drug permeability [39].

After ensuring the absence of air bubbles within the chambers by visual inspection, the permeation cell was placed into a 30±0.1°C waterbath shaking at 120 cycles per minute to ensure solution agitation. Samples of 1 $cm^3$ were withdrawn from the receptor solution at timed intervals and replaced with an equal volume of the appropriate solvent; the dilution was subsequently accounted for when calculating permeation data. Unless assayed immediately, samples were stored at 4°C prior to determination of hyoscine hydrobromide concentration using HPLC (section 2.2.5). The increase in drug concentration within the receptor compartment was monitored for approximately 4 days, during which time the decrease in

donor drug concentration was also routinely monitored.

On completion of permeation experiments, the integrity of the epidermal section was assessed by introducing a small volume of a non-permeating molecule - radiolabelled hyoscine methyl chloride solution into the donor compartment. Appearance of radioactivity, assayed by scintillation counting (section 2.2.3.) in the receptor compartment was taken to be indicative of the presence of perforations or other damage to the membrane and data was discarded. Hyoscine methyl chloride was not found, during the present study, to permeate through intact epidermis to a detectable extent.

Due to difficulties in obtaining cadaver skin, each determination was performed in triplicate only. Skin samples were assigned to particular experiments on a purely random basis.

**7.1.1.3.1. The influence of donor drug solution concentration and pH on the <u>in vitro</u> skin permeation of hyoscine hydrobromide**

Michaels et al. [39] and Chandrasekaran [42] found that hyoscine hydrobromide permeated well through human skin (during <u>in vitro</u> studies) from donor solutions at pH 9.6; drug permeation was found to increase with increasing donor drug concentration, also at pH 9.6.

For this study, hyoscine hydrobromide ($2.28 \times 10^{-2}$ mol $dm^{-3}$) at pH 3.0 and 6.0, using citrate-phosphate buffer, 0.04 M, and at pH 9.7 with 0.04 M borate buffer, were used as donor solutions.

Donor drug solutions of various concentrations: $2.28 \times 10^{-2}$, $1.14 \times 10^{-3}$ and $2.8 \times 10^{-3}$ mol $dm^{-3}$ were prepared by dissolving hyoscine hydrobromide in 0.04 M borate buffer, pH 9.7. Hyoscine hydrobromide flux through human skin was calculated using the method

described in section 1.2.4.3.

### 7.1.2. Determination of the extent of hyoscine hydrobromide loss during storage at pH 3.0 and 9.7

Hyoscine hydrobromide was dissolved in 0.04 M citrate-phosphate buffer, pH 3.0 and 0.04M borate buffer, pH 9.7 to produce drug solutions of $2.28 \times 10^{-4}$ mol $dm^{-3}$. 150 ml of each solution was placed into a volumetric flask, protected from light and kept in a water bath maintained at $30 \pm 0.1^{o}C$. Solutions were assayed for hyoscine hydrobromide using HPLC (section 2.2.5) before, after 4 days, and following storage for 2 weeks, and compared with freshly prepared hyoscine hydrobromide solutions.

### 7.1.3. Buffer pH stability during 3-compartmental electrophoretic drug delivery

The 3-compartmental apparatus (described in section 7.1.) incorporating a 40T 7C polyacrylamide gel disc and full thickness human skin was used to assess pH stability during electrophoretic drug delivery. $1 \times 10^{-3}$M phosphate buffer was used in compartment 3 throughout the determination. Compartments 1 and 2 were buffered at pH's 3.0 and 9.7 respectively but using buffers of equal ionic strength. Citrate-phosphate buffer (chamber 1) and borate buffer (chamber 2) of $1 \times 10^{-3}$M, $4 \times 10^{-2}$M and 1M ionic strengths were used.

After approximately 100 hours of electrophoresis, at a constant 10 volts and with the apparatus maintained at $30 \pm 0.1^{o}C$, the pH of each compartment was determined.

## 7.2. Results and discussion

Drug permeation profiles similar to those expected (section 1.2.4.3.) were obtained during hyoscine hydrobromide skin permeation studies. Lag times varied between 1.4 and 8 hours with shorter lag times generally associated with greatest rates of skin permeation.

Figure 7.4 shows that the rate of *in vitro* permeation of hyoscine hydrobromide increased with increasing donor solution pH. This is similar to the findings of Michaels et al. [39] who observed that unionized hyoscine hydrobromide was nearly 20 times more permeable in human skin than ionized drug. During this study, the greatest and lowest permeation rates were found when hyoscine hydrobromide (pKa 7.35) was either completely in the unionized (pH 9.7) or ionized forms (pH 3.0) respectively. An intermediate permeation rate was found at pH 6.0 when the drug was assumed to be approximately 96% ionized.

Figure 7.3 indicates a linear relationship between donor drug solution concentration and the *in vitro* flux of hyoscine hydrobromide through human skin. Increased drug donor concentration was expected to produce greater drug flux since the diffusive permeation process relies upon the concentration gradient across the membrane, which is greatest at high donor concentrations.

The observed permeation rates are lower than those found by Shaw and Chandrasekaran who studied hyoscine permeation through stomach skin [50] and found permeation rates of approximately $2 \times 10^{-8}$ mol $cm^{-2}$ $hr^{-1}$ compared with $2 \times 10^{-9}$ mol $cm^{-2}$ $hr^{-1}$ obtained during this study. However, their permeation experiments utilized hyoscine free base in mineral oil at a concentration of 4.7 mg $g^{-1}$ as the donor.

Donor drug depletion was found to be greater than expected during

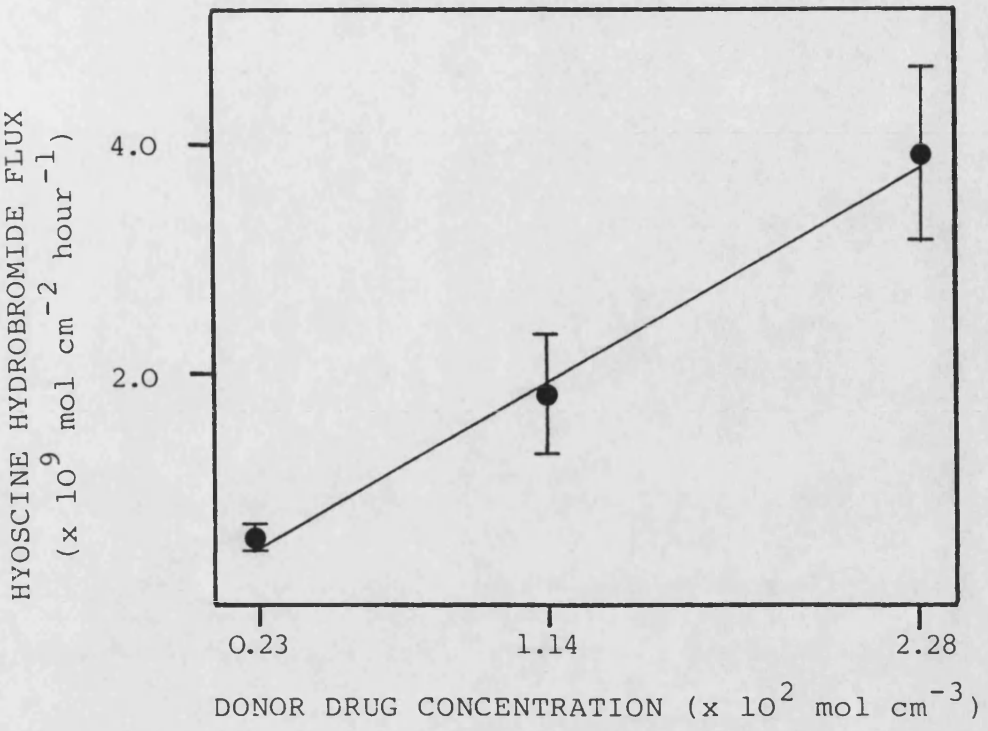



FIGURE 7.3 EFFECT OF DONOR DRUG CONCENTRATION ON IN VITRO HYOSCINE HYDROBROMIDE FLUX THROUGH HUMAN ABDOMINAL EPIDERMIS AT pH 9.7

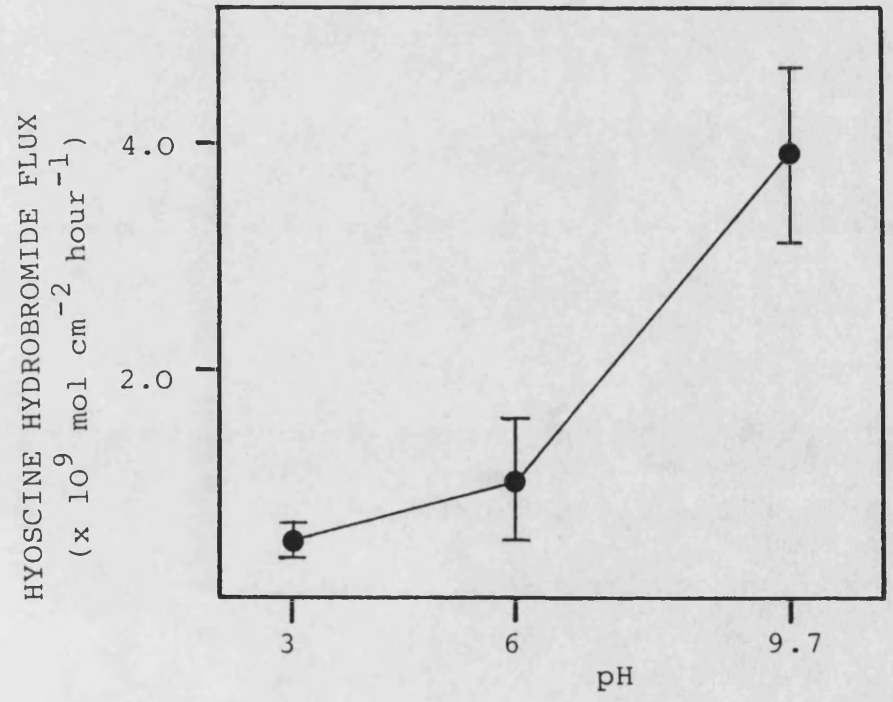



FIGURE 7.4 EFFECT OF DONOR pH ON IN VITRO HYOSCINE HYDROBROMIDE FLUX THROUGH HUMAN ABDOMINAL EPIDERMIS

permeation experiments at pH 9.7.

Storage of hyoscine hydrobromide solution at pH 3.0 for 2 weeks resulted in no detectable drug loss. However, approximately 90% of drug remained after 4 days with approximately 60% remaining after 2 weeks of storage at pH 9.7. Hyoscine hydrobromide instability in solution has been reported; the free bases of alkaloids are considered to be unstable [216].

Thus the excessive donor drug depletion found during this study was probably due to hyoscine hydrobromide breakdown at pH 9.7. Although different buffers may have been used, Michaels et al. [39] did not discuss the possibility of donor drug degradation and in fact stated that donor drug concentration was not routinely monitored during hyoscine hydrobromide permeation experiments.

No degradants were detected during HPLC assay for hyoscine hydrobromide and the possibility of drug depletion by adsorption onto vessel walls was not eliminated. Whatever the mechanism of donor drug loss, the results of hyoscine hydrobromide skin permeation obtained in the present study cannot be utilised as absolute values.

In studies of pH stability of buffers intended for 3-compartmental delivery investigations, the pH in compartment 3 remained unchanged after electrophoresis runs. There was a decrease of approximately 1 pH unit in compartment 1 (anolyte) and an increase of between 2 and 3 pH units in compartment 2 (catholyte). A previously observed trend of increased effectiveness of buffer with increasing buffer ionic strength (section 6.A.1.) was not found. The directions of pH changes observed were as expected from consideration of electrolytic processes occurring (section 1.5.4.4.v.) during

electrophoresis but the buffering arrangement utilised in this study probably gave rise to complex electrochemical reactions which manifested as the observed pH changes.

Hyoscine hydrobromide was shown to permeate through human skin *in vitro*. The rate of permeation was dependent upon donor drug solution concentration and pH. However conditions of pH favourable to drug permeation appeared to be detrimental to drug stability. Adequate buffering of the 3-compartmental model for studying electrophoretic drug delivery to skin was not achieved and pH changes, probably due to electrolysis reactions were not reduced by alteration of buffer ionic strengths.

# CONCLUSIONS

## CONCLUSIONS

Polyacrylamide gel rods, discs and sheets and polyHEMA gel rods could be prepared by chemically initiated polymerization using the methods described in this study. However, neither polyacrylamide or polyHEMA gels of an acceptable quality were produced by gamma-irradiation initiated polymerization during this study due to inadequate temperature control and lack of maintenance of monomer deaeration during preparation.

Rod polyacrylamide gel electrophoresis (PAGE) indicated that lower voltages than normally utilised in conventional PAGE techniques could be used to produce reproducible migration of proteins such as bovine serum albumin (BSA) and insulin. The electrophoretic migration of these proteins was influenced by polyacrylamide gel formulation, magnitude of applied voltage (field strength), temperature and electrolyte/gel buffer ionic strength and pH.

Rod gel electrophoresis using polyHEMA gel was unsuccessful for the electrophoresis of BSA and insulin; the electrophoretic passage of these molecules was probably restricted by the relatively small pore size of polyHEMA gels used compared with polyacrylamide gels employed.

Electron microscopy studies suggested that a heterogeneous polyacrylamide gel structure existed in the micron range; thus it was considered that any molecular sieving must occur within an infrastructure comprising pores approaching molecular dimensions.

Polyacrylamide gels were found to swell in water and citrate-phosphate buffer solutions but reached an equilibrium swelling after

approximately 100 hours. The extent of polyacrylamide gel swelling was found to be influenced by polymerization temperature and gel formulation factors such as total monomer concentration and percentage crosslinking together with the ionic strength of the immersing buffer. Swelling of polyacrylamide gel was found to be negligible during electrophoresis.

U.V. absorbing materials, believed to be unreacted monomers and catalysts, were leached from polyacrylamide gels(produced by chemically initiated polymerization)during storage in buffer solution; the release rate of contaminants was high for the first 2 days and then decreased markedly on further storage.

A "solution depletion" technique used in this study to determine drug partition coefficients in polyacrylamide gels was unsuccessful; a source of interference may have been microbial contamination.

Hyoscine hydrobromide, propranolol hydrochloride, hyoscine methyl chloride and insulin were found to permeate through polyacrylamide gel, probably via a "pore mechanism". The permeation of these drugs was found to be influenced by polyacrylamide gel formulation, decreasing as gel monomer concentration was increased and showing a minimum at 6-8% crosslinking (C) with Bis.

The permeation of the above drugs may be minimized by manipulation of polyacrylamide gel formulation and by increasing gel thickness. Thus polyacrylamide gel discs were used to provide a barrier to drug permeation from a reservoir solution.

By application of an electric field, drugs of both high molecular weight such as insulin and low molecular weight such as hyoscine hydrobromide, propranolol hydrochloride and hyoscine methyl chloride, could be eluted from the reservoir solution. By use of a stabilized power supply to provide constant low voltage conditions (5 and 10 volts), the above drugs were released at constant rates (zero-order) for several days. In addition, insulin and hyoscine methyl chloride were found to be released in a reproducible manner, intermittently, by removal and re-application of the electric field. Drug release rates were found to be affected by the strength of electric field applied, temperature and polyacrylamide gel formulation. Ionic migration and electrolysis gave rise to pH changes during electrophoretic drug release .

Hyoscine hydrobromide was found to permeate through human skin in vitro, the permeation rate was influenced by donor drug solution pH and concentration. However, at pH 9.7 (which was favourable to skin permeation) hyoscine hydrobromide loss (probably through degradation) occurred.

A three-compartmental model intended for the study of controlled in vitro hyoscine hydrobromide delivery to human skin was not completely successful due to problems of drug stability and pH drift during electrophoresis.

**Suggestions for further work**

Polyacrylamide gel was found to be permeable to drugs used in this study. Investigation of alternative polymers capable of preventing drug permeation yet permitting electrophoretic drug elution may lead to improved control of drug release; in addition, absence of residual chemicals and minimal or no swelling in use would be desirable properties.

Constant voltage power supplies were used throughout this study. However, a constant current source with microprocessor control to produce currents in the micro- and milliamp range was constructed but not validated. Electrophoretic drug release studies using this constant current source may produce more reproducible drug release than found in the present study by overcoming the effects of changes in electrical resistance within the electrophoretic apparatus.

The problem of pH control during this study requires further study and may be reduced or eliminated by the use of alternative electrodes and buffer systems for electrophoresis.

**BIBLIOGRAPHY**


1. GIBALDI, M. (1977)
Biopharmaceutics and Pharmacokinetics. Philadelphia: Lea and Febinger. Page 1.

2. GANDERTON, D. (1985)
Manf. Chem., March: 27

3. LANGER, R. (1980)
Chem. Eng. Commun., 6:1

4. CHANDRASEKARAN, S.K., WRIGHT, R.M. and YUEN, M.J. (1983)
Controlled release: Status and future prospects. In: Controlled release delivery systems (Roseman, T.J. and Mansdorf, S.Z., Eds.). New York: Marcel Dekker.

5. MICHAELS, A.S. (1974)
Therapeutic systems for controlled administration of drugs. In: Permeability of plastics, films and coatings to gases, vapours and liquids (Hopfenberg, H.B., Ed.). New York: Plenum Press.

6. DONBROW, M. and SAMUELOV, Y. (1980).
J. Pharm. Pharmacol., 32:463

7. WOOD, J.M. (1983)
A study of the properties of poly(HEMA) hydrogels for use in controlled-release drug delivery systems. PhD Thesis, Victoria University of Manchester.

8. JOHNSON, J.C., Ed. (1980)
Sustained release medications. New Jersey: Noyes Data Corporation.

9. ROSEMAN, T.J. and MANSDORF, S.Z., Eds. (1983)
Controlled release delivery systems. New York: Marcel Dekker,

10. GOLDMAN, P. (1982)
N. Engl.J. Med., 307:286

11. URQUHART, J. (1981)
Controlled release pharmaceuticals. Washington: American Pharmaceutical Association.

12. SIEGEL, R.A. and LANGER, R. (1983)
Pharm. Res., 2: 1

13. COWSAR, D.R. (1975)
Drug delivery systems: Design criteria. In: Polymers in medicine and surgery (Kronenthal, R.L., Oser, Z. and Martin, E., Eds.). New York: Plenum Press.

14. JULIANO, R.L., Ed. (1980)
Drug delivery systems. New York: Oxford University Press.

15. ROBINSON, J.R., Ed. (1978)
Sustained and controlled drug delivery systems. New York: Marcel Dekker.

16. HEILMANN, K. (1984)
Therapeutic systems. Stuttgart, West Germany: Ferdinand Enke Verlag.

17. PAUL, D.R. and HARRIS, F.W., Eds. (1976)
Controlled release polymeric formulations. Washington, D.C.: American Chemical Society.

18. ANDERSON, J.M. and KIM, S.W., Eds. (1983)
Recent advances in drug delivery systems. New York: Plenum Press.

19. HEILMANN, K. (1984)
Therapeutic systems. Stuttgart, West Germany: Ferdinand Enke Verlag. Page 67.

20. COWSAR, D.R., TARWATER, O.R. and TANQUARY, A.C. (1976)
Controlled release of fluoride from hydrogels for dental applications. In: Hydrogels for medical and related applications (Andrade, J.D., Ed.). Washington, D.C.: American Chemical Society.

21. POUTON, C.W. (1985)
J. Clin. & Hosp. Pharm., 10:45

22. GARDNER, C.R, (1985)
Biomaterials, 6:153

23. FOLKMAN, J. and LONG, D.M. (1964)
J. Surg. Res., 4 (3):139

24. DAVIS, B.K. (1972)
Experientia, 28 (1):348

25. CREQUE, H.M., LANGER, R. and FOLKMAN, J. (1980)
Diabetes, 29:37

26. WOOD, D.A. (1980)
Int. J. Pharm., 7:1

27. KOCH-WESER, J. and SCHECHTER, P.J. (1979)
Slow-release preparations in clinical perspective. In: Drug Absorption. Proc. Int. Conf. (Prescott, L.F. and Nimmo, W.S., Eds.). Lancaster: MTP Press Ltd.

28. THEEUWES, F. (1979).
Novel drug delivery systems. In: ibid.

29. MORIMOTO, K., TAKEEDA, T., NAKAMOTO, Y. and MORISAKA, K. (1982)
Int. J.Pharm., 12:107

30. MORIMOTO, K., MORISAKA, K. and KAMADA, A (1985)
J. Pharm. Pharmacol., 37:134

31. SU, K.S.E., CAMPANALE, K.M., MENDELSOHN, L.G., KERCHNER, G.A. and GRIES, C.L. (1985)
J. Pharm. Sci., 74(4):394

32. WEST, G.B. (1982)
Chemist & Druggist:496

33. ICHIKAWA, K., OHATA, I., MITOMI, M., KAWAMURA, S., MAENO, H. and KAWATA, H. (1980)
J. Pharm. Pharmacol., 32:314

34. LIVERSIDGE, G.G., NISHIHATA, T., ENGLE, K.K. and HIGUCHI, T. (1985)
Int. J. Pharm., 23:87

35. NISHIHATA, T., RYTTING, J.H., HIGUCHI, T. and CALDWELL, L. (1981)
J. Pharm. Pharmacol., 33:334

36. MORIMOTO, K., KAMIYA, E., TAKEEDA, T., NAKAMOTO, Y. and MORISAKA, K. (1983)
Int. J. Pharm., 14:149

37. KIM, S., KAMADA, A., HIGUCHI, T. and NISHIHATA, T. (1983)
J. Pharm. Pharmacol., 35:100

38. DE BOER, A.G. and BREIMER, D.D. (1979)
Rectal absorption: Portal or systemic? In: Drug absorption. Proc. Int. Conf. (Prescott, L.F. and Nimmo, W.S., Eds.). Lancaster:MTP Press Ltd.

39. MICHAELS, A.S., CHANDRASEKARAN, S.K. and SHAW, J.E. (1975)
AIChE.J., 21 (5):985

40. BLANK, I.H. (1964)
J. Invest. Dermatol., 43:415

41. SCHWARTZEL, R.J. (1967)
J. Invest. Dermatol., 48 (1):79

42. CHANDRASEKARAN, S.K. (1980)
Drug permeation through skin: Controlled delivery for topical or systemic therapy. In: Advances in biomedical engineering (Cooney, D.O., Ed.). New York: Marcel Dekker.

43. FELDMANN, R.J. and MAIBACH, H.I. (1970)
J. Invest. Dermatol., 54 (5):399

44. FEIWEL, M., JAMES, V.H.T. and BARNETT, E.S. (1969)
Lancet:485

45. KLIGMAN, A.M. (1978)
Curr. Probl. Dermatol., 7:1

46. BARRY, B.W. (1983)
Properties that influence percutaneous absorption. In:
Dermatological formulations. New York: Marcel Dekker Inc

47. SCHLEUPEIN, R.J. and BLANK, I.H. (1971)
Physiol. Rev., 51 (4):702

48. FELDMAN, R.J. (1967)
J. Invest. Dermatol., 48:181

49. BARTEK, M.J., LA BUDDLE, J.A. and MAIBACH, H.I. (1972)
J. Invest. Dermatol., 58:114

50. SHAW, J.E. and CHANDRASEKARAN, S.K. (1979)
Transdermal therapeutic systems. In: Drug absorption.
Proc. Int. Conf. (Prescott, L.F. and Nimmo, W.S., Eds.).
Lancaster: MTP Press Ltd.

51. PRICE, N.M., SCHMITT, L.G., McGUIRE, J., SHAW, J.E. and
TROBOUGH, G. (1981)
Clin. Pharmacol. Ther, 29 (3): 414

52. SHAW, J.E. and DOHNER, J.W. (1985)
Manf. Chem., May: 53

53. SHAW, J.E. and URQUHART, J. (1980)
TIPS: 208

54. SHAW, J.E. and URQUHART, J. (1981)
Br. Med. J., 283:875

55. BICKERS, D.R. (1980)
The skin as a site of drug and chemical metabolism. In: Current
concepts in cutaneous toxicity (Drill, V.A. and Lazar, P.,
Eds.). New York: Academic Press.

56. McDONALD, H.J. (1955)
Ionography: electrophoresis in stabilized media. Chicago: Year
Book Publishers Inc. Page 25.

57. MARTIN, A.N., SWARBRICK, J. and CAMMARATA, A. (1969)
Physical Pharmacy. Philadelphia: Lea and Febinger. Page 190.

58. WALTERS, K.A. (1985)
Personal communication.

59. PATEL, N.K. and FOSS, N.E. (1964)
J. Pharm. Sci., 53 (1):94

60. NUGENT, F.J. and WOOD, J.A. (1980)
Can. J. Pharm. Sci., 15 (1):1

61. CHOWHAN, Z.T. and PRITCHARD, R. (1978)
J. Pharm. Sci., 67 (9):1272

62. COOPER, E.R. and BERNER, B. (1985)
Skin permeability. In: Methods in skin research (Skerrow, D. and Skerrow, C.J., Eds.). Great Britain: John Wiley and Sons Ltd.

63. SWARBRICK, J., LEE, G. and BROM, J. (1982)
J. Invest. Dermatol., 78(1):63

64. BARRY, B.W. (1983)
Basic principles of diffusion through membranes. In: Dermatological formulations. New York: Marcel Dekker.

65. FRANZ, T.J. (1978)
Curr. Probl. Dermatol., 7:58

66. ANONYMOUS (1982)
Pharm. J. October:472

67. GUY, R.H. and HADGRAFT, J. (1985)
Pharm. Int. May:112

68. BERNER, B. (1985)
J. Pharm. Sci., 74 (7):718

69. GUY, R.H. and HADGRAFT, J. (1985)
Int. J. Pharm., 24:267

70. SHAW, J.E., SCHMITT R.N., McCAULEY, M.E. and ROYAL, J.W. (1977)
Clin. Pharmacol. Ther., 21 (1):117

71. HURKMANS, J.F.G.M., BODDE, H.E., VAN DRIEL, L.M.J., VAN DOORNE, H. and JUNGINGER, H.E. (1985)
Br. J. Dermatol., 112:461

72. ANDRADE, J.D., Ed. (1976)
Hydrogels for medical and related applications. Washington D.C.: American Chemical Society.

73. WOOD, D.A. (1984)
Polymeric materials used in drug delivery systems. In: Materials used in pharmaceutical formulation (Florence, A.T., Ed.). Oxford: Blackwell Scientific Publications.

74. SEYMOUR, R.B. and CARRAHER, C.E. (1981)
Step-reaction polymerization or polycondensation reactions. In: Polymer chemistry - an introduction. New York: Marcel Dekker.

75. HSIEH, D.S.T., RHINE, W.D. and LANGER, R. (1983)
J. Pharm. Sci., 72 (1):17

76. SCHWOPE, A.D., WISE, D.L. and HOWES, J.F. (1975)
Life Sciences, 17:1877

77. EDELMAN, E.R., KOST, J., BOBECK, H. and LANGER, R. (1985)
J. Biomed. Mater. Res., 19:67

78. WICHTERLE, O. and LIM, D. (1960)
Nature, 185:117

79. SCHACHT, E.H. (1984)
Hydrogel drug delivery systems. Physical and ionogenic drug carriers. In: Recent advances in drug delivery systems. (Anderson, J.M. and Kim, S.W., Eds.). New York:Plenum Press.

80. MACK, E.J., SHARMA, K. and KIM, S.W. (1983)
Hydrogel diffusion and drug delivery systems. In: Proc. 43rd Int. Congr. Pharm. Sci. (Breimer, D.D. and Speiser, P., Eds.). Amsterdam: Elsevier Science Publishers.

81. DROBNIK, J., SPACEK, P. and WICHTERLE, O. (1974)
J. Biomed. Mater. Res., 8:45

82. ZENTNER, G.M., CARDINAL, J.R. and KIM, S.W. (1978)
J. Pharm. Sci., 67 (10):1352

83. GOOD, W.R. (1978)
Diffusion of water soluble drugs from initially dry hydrogels. In: Polymeric delivery systems (Kostelnik, R. J., Ed.). London: Gordon and Breach Science Publishers.

84. SEYMOUR, R.B. and CARRAHER, C.E. (1981)
Testing and characterization of polymers. In: Polymer chemistry - an introduction. New York: Marcel Dekker.

85. WOOD, J.M., ATTWOOD, D. and COLLETT, J.H. (1983)
Drug Devel. Ind. Pharm., 9:93

86. DAVIS, B.K. (1974)
Proc. Nat. Acad. Sci. USA, 71 (8):3120.

87. KOPACEK, J., SPRINCL, L., BAZILOVA, H. and VACIK, J. (1973)
J. Biomed. Mater. Res., 7:111

88. SPRINCL, L., KOPACEK, J. and LIM, D. (1973)
Cal. Tiss. Res., 13:63

89. BRUCK, S.D. (1973)
J. Biomed. Mater. Res., 7:387

90. LANGER, R. and FOLKMAN, J. (1976)
Nature, 263:797

91. JHON, M.S. and ANDRADE, J.D. (1973)
J. Biomed. Mater. Res., 7:509

92. LEE, H.B., JHON, M.S. and ANDRADE, J.D. (1974)
J. Coll. Interf. Sci., 51 (2):225

93. BARRER, R.M. (1951)
Diffusion in and through solids. Cambridge. Through: Bray, C.S. (1977). MSc. Thesis. University of Bath.

94. KUMINS, C.A. and KWEI, T.K. (1968)
Free volume and other theories. In: Diffusion in polymers (Crank, J. and Park, G.S., Eds.). London: Academic Press.

95. CHIEN, Y.W. (1980)
Controlled drug release from polymeric delivery systems: biomedical applications and physicochemical prin ciples. In: Drug delivery systems (Juliano, R.L., Ed.). New York: Oxford University Press.

96. BAKER, R.W., TUTTLE, M.E., LONSDALE, H.K. AND AYRES, J.W. (1979)
J. Pharm. Sci., 68 (1):20

97. BEAL, M. (1983)
Polyamide microcapsules as a basis for the treatment of glaucoma. PhD Thesis, University of Bath.

98. HIGUCHI, T. (1963)
J. Pharm. Sci., 52 (12):1145

99. FLYNN, G.L. et al. (1976)
Interfacing matrix release and membrane absorption - Analysis of steroid absorption from a vaginal device in the rabbit doe. In: Controlled release polymeric formulations. (Paul, D.R. and Harris, F.W., Eds.). Washington, D.C.: American Chemical Society.

100. CHIEN, Y.W. (1976)
Thermodynamics of controlled drug release from polymeric delivery devices. In: ibid.

101. KUU, W., YALKOWSKY, S.H. (1985)
J. Pharm. Sci., 74 (9):926

102. ROWE, R.C. (1975)
Manf. Chem. Aerosol. News, 46 March: 23

103. RHINE, W.D., HSIEH, D.S.T. and LANGER, R. (1980)
J. Pharm. Sci., 69 (3):265

104. MUHR, A.H. and BLANSHARD, J.M.V. (1982)
Polymer, 23:1012

105. YASUDA, H., LAMAZE, C.E. and IKENBERRY, L.D. (1968)
Die. Makromol. Chem., 118:19

106. ZENTNER, G.M., CARDINAL, J.R., FEIJEN, J. and SONG, S. (1979)
J. Pharm. Sci., 68 (8):970

107. WOOD, J.M., ATTWOOD, D. and COLLETT, J.H. (1981)
J. Pharm. Pharmacol., 34:1

108. WISNIEWSKI, S.J., GREGONIS, D.E., KIM, S.W. and ANDRADE, J.D. (1976)
Diffusion through hydrogel membranes. In: Hydrogels for medical and related applications (Andrade, J.D., Ed.). Washington, D.C.: American Chemical Society.

109. LEE, P.I. (1980)
J. Membr. Sci., 7:255

110. PEPPAS, N.A., GURNY, R., DOELKER, E. and BURI, P. (1980)
J. Membr. Sci., 7:241

111. MARTIN, A.N., SWARBRICK, J. and CAMMARATA, A. (1969)
Physical Pharmacy. Philadelphia: Lea and Febinger. Page 161.

112. THEEUWES, F. and HIGUCHI, T. (1974)
Alza Corporation. U.S. Pat. 3,845,770

113. HEILMANN, K. (1984)
Therapeutic systems. Stuttgart, West Germany: Ferdinand Enke Verlag. Page 63.

114. SMITH, K.L., SHAH, A.C., ALBERT, K.S., SZPUNAR, G.Z. and PRATT, E.A. (1985)
Proceed. Intern. Symp. Control. Rel.Bioact. Mater., 12:231

115. BREIMER, D.D., DE BOER, A.G. and DE LEEDS, L.G.J. (1985)
ibid: 227

116. LANGER, R. (1982)
Chemtech, 2:98

117. HSIEH, D.S.T. and LANGER, R. (1983)
Zero-order drug delivery systems with magnetic control. In: Controlled release delivery system. (Roseman, T.J. and Mansdorf, S.Z., Eds.). New York: Marcel Dekker.

118. LANGER, R., BROWN, L. and EDELMAN, E. (1984)
Controlled release and magnetically modulated systems for macromolecules: Recent advances. In: Recent advances in drug delivery systems. (Anderson, J.M. and Kim, S.W., Eds.). New York: Plenum Press.

119. UDENFRIEND, S., STEIN, S., BOHLEN, P. and DAIRMAN, W. (1972)
Science, 178:871.

120. BOULTON, A.J.M., DRURY, J., CLARKE, B. and WARD, J.D. (1982)
Diabetes Care, 5 (4): 386

121. CLEMENS, A.H. (1979)
Med. Progr. Technol., 6:91

122. SCHADE, D.S., et al. (1981)
Diabetes Care, 4 (2): 319

123. SEFTON, M.V., ALLEN, D.G., HORVATH, V. and ZINGG, W. (1984)
Insulin delivery at variable rates from a controlled release micropump. In: Recent advances in drug delivery systems (Anderson, J.M. and Kim, S.W., Eds.). New York: Plenum Press.

124. BUCHWALD, H., ROHDE, T.D., SCHNEIDER, P.D., VARCO, R.L. and BLACKSHEAR, P.J. (1980)
Surgery, 88 (4):507

125. GANGAROSA, L.P. and PARK, N.H. (1978)
J. Prosthet. Dent., 39 (2):173

126. GANGAROSA, L.P., PARK, N.H., FONG, B.C., SCOTT, D.F. and HILL, J.M. (1978).
J. Pharm. Sci., 67(10):1439

127. LEVCHENKO, N.F., DANILOVA, I.N. and SHARPANOVA, I.K. (1981)
Vopr. Kuroktol Fizioter Lech. Fiz. Kult, 1:49

128. SIDDIQUI, O., ROBERTS, M.S. and POLACK, A.E. (1985)
J. Pharm. Pharmacol., 37:732

129. GLASS, J.M., STEPHEN, R.L. and JACOBSON, S.C. (1980)
Int. J. Dermatol., 19:519

130. ANONYMOUS (1985)
Manuf. Chem., April: 53

131. VOLD, R.D. and VOLD, M.J. (1983)
Electrical characteristics of lyophobic sols. In: Colloid and interface chemistry. Massachusetts: Addison-Wesley Publishing Company.

132. SHAW, D.J. (1969)
Electrophoresis. London: Academic Press. Page 1.

133. POPIEL, W.J. (1978)
Optical and kinetic properties of colloids. In: Introduction to colloid science. New York: Exposition Press.

134. MARTIN, A.N., SWARBRICK, J. and CAMMARATA, A. (1969)
Interfacial phenomena. In: Physical Pharmacy. Philadelphia: Lea and Febinger.

135. MAURER, H.R. (1971)
Theoretical background. In: Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis. Berlin: de Gruyter.

136. GAAL, O., MEDGYESI, G.A. and VERECZKEY, L. (1980)
Electrophoresis in the separation of biological macromolecules. Budapest: Akademiai Kiado. Page 15.

137. HÜCKEL, E. (1924)
Phys. Z., 25:204. Through: Shaw, D.J. (1969). Electrophoresis. London: Academic Press. Page 16.

138. SMOLUCHOWSKI, M. (1914)
In: Handbuch der Eleckrizitat und des Magnetismus. Leipzig, Germany. Through: Shaw, D.J. ibid.

139. HENRY, D.C. (1931)
Proc. Res. Soc., A133:106. Through: Shaw, D.J. ibid.

140. TISELIUS, A. (1937)
Trans. Farad. Soc., 33: 524

141. GAAL, O., MEDGYESI, G.A. and VERECZKY, L. (1980)
Electrophoresis in the separation of biological macromolecules. Budapest: Akademiai Kiado. Page 37.

142. RILBE, H. (1983)
Basic theory of electrophoresis: definitions, terminology, and comparison of the basic techniques. In: Electrophoretic techniques (Simpson, C.F. and Whittaker, M., Eds.). London: Academic Press.

143. Consden, R., Gordon, A.H. and Martin, A.J.P. (1946)
Biochem. J., 40:33

144. SMITHIES, O. (1955)
Biochem. J., 61:629

145. RAYMOND, S. and WEINTRAUB, L. (1959)
Science, 130: 711

146. JORDON, E.M. and RAYMOND, S. (1969)
Anal. Biochem., 27:205

147. BIO-RAD (1984)
Acrylamide polymerization - A practical approach. BIO-RAD Bulletin 1156. Bio-Rad Laboratories.

148. BREWER, J.M. (1967)
Science, 156:256

149. FANTES, K.H. and FURMINGER, I.G.S. (1967)
Nature, 215:750

150. PETERSON, R.C. (1967)
J. Pharm. Sci., 56 (11): 1489

151. GRESSEL, J., ROSNER, A. and COHEN, N. (1975)
Anal. Biochem., 69:84

152. GORDON, A.H. (1973)
Electrophoresis of proteins in polyacrylamide and starch gels. In: Laboratory techniques in biochemistry and molecular biology (Work, T.S. and Work, E., Eds.). New York: American Elsevier.

153. HJERTEN, S. (1962)
Arch. Biochem. Biophys. Suppl., 1:147

154. HAMES, B.D. (1981)
An introduction to polyacrylamide gel electrophoresis. In: Gel electrophoresis of proteins: a practical approach. Oxford: IRL Press Ltd.

155. CHRAMBACH, A. and RODBARD, D. (1971)
Science, 172:440

156. HEDRICK, J.L. and SMITH, A.J. (1968)
Arch. Biochem. Biophys., 126:155

157. SMITHIES, O. (1962)
Arch. Biochem. Biophys. Suppl., 1:125

158. ORNSTEIN, L. (1964)
Annals. N.Y. Acad. Sci., 121:321

159. RAYMOND, S. and NAKAMICHI, M. (1962)
Anal. Biochem., 3:23

160. TOMBS, M.P. (1968).
Acrylamide flat gel electrophoresis. In: Chromatographic and electrophoretic techniques. Vol. 2 (Smith, I., Ed.). London: W.Heinemman Medical Books.

161. TOMBS, M.P. (1965)
Anal. Biochem., 13:121

162. HJERTEN, S. (1967)
Prot. Biol. Fluids., 14: 553

163. MORRIS, C.J.O.R. and MORRIS, P. (1971)
Biochem. J., 124:517

164. RODBARD, D. (1976)
Estimation of molecular weight by gel filtration and gel electrophoresis. 1. Mathematical principles. In: methods of protein separation Vol. 2. (Catsimpoolas, N., Ed.). New York: Plenum press.

165. FISHER, M.P. and DINGMAN, C.W. (1971).
Biochemistry, 10 (10):1895

166. WAGMAN, G.H. and WEINSTEIN, M.J. (1973)
Chromatography of antibiotics. Amsterdam: Elsevier Scientific Publishing Company.

167. RODBARD, D., LEVITOV, C. and CHRAMBACH, A. (1972)
Separat. Sci., 7 (6):705

168. HSU, T.T.P. (1983)
Thermodynamics and network structure of polyacrylamide gels. PhD Thesis. Cornell University, New York.

169. BAUMANN, G. and CHRAMBACH, A. (1976)
Anal. Biochem., 70:32

170. BLANK, Z. and REIMSHUESSEL, A.C. (1974)
J. Mater. Sci., 9:1815

171. RUCHEL, R. and BRAGER, M.D. (1975)
Anal. Biochem., 68:415

172. RUCHEL, R., STEERE, R.L. and ERBE, E.F. (1978)
J. Chromatogr., 166:563

173. CALVERT, P. (1975)
Nature, 254:104

174. BOYDE, T.R.C. (1976)
J. Chromatogr., 124:219

175. RICHARDS, E.G. and LECANIDOU, R. (1974)
Polymerization kinetics and properties of polyacrylamide gels. In: Electrophoresis and isoelectric focusing in polyacrylamide gel. (Allen, R.C. and Maurer, H.R., Eds.). Berlin: De Gruyter.

176. RICHARDS, E.G. and TEMPLE, C.J. (1971)
Nature Phys. Sci., 230:92

177. JANAS, V.F., RODRIGUEZ, F. and COHEN, C. (1980)
Macromolecules, 13(4):977

178. FERGUSON, K.A. (1964)
Metabolism, 13 (10):985

179. PHARMACIA. (1982)
Isoelectric focusing. Uppsala, Sweden: Pharmacia Fine Chemicals.

180. ANDREWS, A.T. (1981)
Electrophoresis: theory, techniques, and biochemical and clinical applications. Oxford: Clarendon press. Pages 5-18

181. ANDREWS, A.T. (1981)
ibid. Page 22-47

182. DAVIS, B.J. (1964)
Annals. N.Y. Acad. Sci., 121:404

183. GAAL, O., MEDGYESI, G.A. and VERECZKEY, L. (1980)
Electrophoresis in the separation of biological macromolecules. Budapest: Akademiai. Kiado. Page 168

184. CHRAMBACH, A., JOVIN, J.M., SVENDSEN, P.J. and RODBARD, D. (1976)
Analytical and preparative gel electrophoresis. In: Methods of protein separation, Volume 2. (Catsimpoolas, N., Ed.). New York: Plenum Press.

185. OSTROWSKI, W. (1979)
Zone electrophoresis. In: Electrophoresis, a survey of techniques and applications. A: techniques (Deyl, Z., Ed.). Amsterdam: Elsevier Scientific Publishing Company.

186. UZGIRIS, E.E. (1974)
Rev. Sci. Instrum., 45 (1):74

187. PALIN, G.R. (1969)
Electrochemistry for technologists. Oxford: Pergamon Press Ltd., Page 20.

188. ENG, P.R. and PARKES, C.O. (1974).
Anal. Biochem., 59:323

189. MacKENZIE, C. (1967)
Experimental Organic Chemistry. New Jersey: Prentice-Hall Inc. page 147.

190. BENNETT, T.P. (1968)
Graphic biochemistry: chemistry of biological molecules. New York: MacMillan Company. Page 679.

191. GEIGY SCIENTIFIC TABLES (1970)
Edited by Diem, K. and Lentner, C. 7th ed. Macclesfield: Geigy Pharmaceuticals.

192. ADAMS, M.J. et al. (1969)
Nature, 224:491

193. MARTINDALE. The Extra Pharmacopoeia (1977)
Edited by Wade, A. 27th ed. London: Pharmaceutical Press.

194. PHARMACEUTICAL CODEX (1979).
London: Pharmaceutical Press.

195. RICHENS, E. (1984)
Personal communication.

196. RODBARD, D. and CHRAMBACH, A. (1974)
Quantitative polyacrylamide gel electrophoresis: mathematical and statistical analysis of data. In: Electrophoresis and isoelectric focusing in polyacrylamide gel (Allen, R.C. and Maurer, H.R., Eds.). Berlin: De Gruyter.

197. YAMADA, S., NODA, N., HAYAKAWA, J., and UNO, K. (1984)
Yakugaku Zasshi, 104 (2):199

198. WOOD, J.M., ATTWOOD, D. and COLLETT, J.H. (1981)
Int. J. Pharm., 7:189

199. AHMED, A.H. (1983)
The effect of sensitizers on inactivation and mutation induction by gamma radiation in Escherichia coli. PhD Thesis, University of Bath.

200. SEFTON, M.V. and NISHIMURA, E. (1980)
J. Pharm. Sci., 69(2):208

201. LEE, K.H., JEE, J.G., JHON, M.S. and REE, T. (1978)
J. Bioeng., 2: 269

202. HJERTEN, S., JERSTEDT, S. and TISELIUS, A. (1965)
Anal. Biochem., 11:211

203. TABER, H.W. and SHERMAN, F. (1964)
Annals. N.Y. Acad. Sci., 121:600

204. WHITE, M.L. and DORION, G.H. (1961)
J. Polym. Sci., 55:731

205. FAWCETT, J.S. and MORRIS, C.J.O.R. (1966)
Separat. Sci., 1 (1):9.

206. LEONING, U.E. (1967)
Biochem. J., 102:251

207. ELVING, P.J., MARKOWITZ, J.M. and ROSENTHAL, I. (1956)
Anal. Chem., 28 (7):1179

208. DANSON, M.J. (1980)
Polyacrylamide gel electrophoresis. Biochemistry practical schedule, University of Bath.

209. DUNKER, A.K. and RUECKERT, R.R. (1969)
J. Biol. Chem., 244: 5074

210 MORRIS, C.J.O.R. and MORRIS, P. (1963)
Separation methods in biochemistry. London: Sir Isaac Pitman & Sons Ltd. Page 639.

211. GROSS, D. (1965)
Analyst, 90:380

212. ABSOLOM, D.R., NEUMANN, A.W. and VAN OSS, C.J. (1984)
Steric considerations in protein adsorption. In: Polymer adsorption and dispersion stability. (Goddard, E.D. and Vincent, B., Eds.). Washington, D.C.: American Chemical Society.

213. THORUN, W. (1971)
Z. Klin. Chem. U. Klin. Biochem., 9:3. Through: Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis (Maurer, H.R.). Berlin: de Gruyter.

214. REFOJO, M.F. (1965)
J. Appl. Polym. Sci., 9:3417

215. BARRY, B.W. (1983)
Methods for studying percutaneous absorption In: Dermatological formulations. New York: Marcel Dekker.

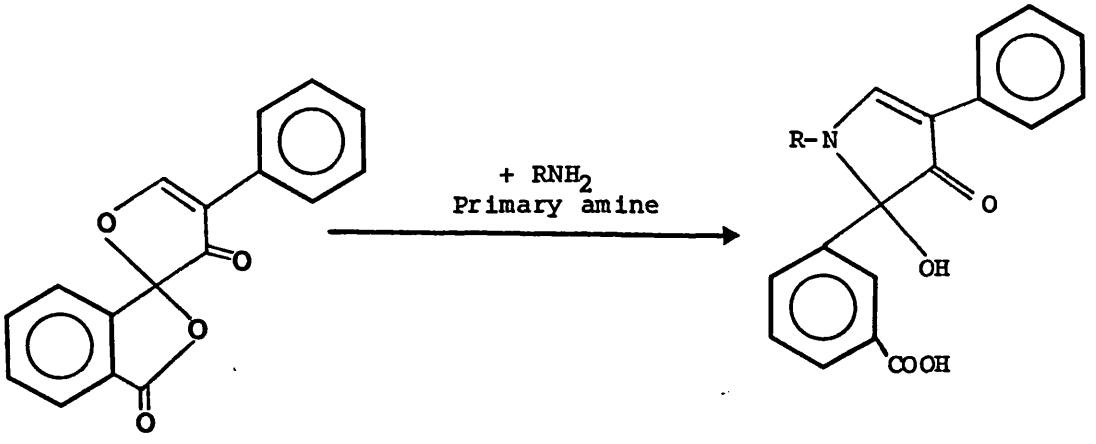
216. PENNINGTON, L.J. and SCHMIDT, W.F. (1982)
J. Pharm. Sci., 71 (8): 951

217. ANONYMOUS (1986)
Pharm.J., June: 764

**Appendix 1**

**The labelling of proteins for electrophoresis with a fluorescent marker**

Fluorescamine was first used as a fluorescent marker for the detection of primary amines by Udenfriend et al. [119] based on its chemical properties. The reaction of fluorescamine with primary amines is completed within a few seconds at room temperature (pH > 7). In addition, fluorescamine and its breakdown products are non-fluorescent.




Fluorescamine Fluorophor

A method similar to that first described by Eng and Parkes [188] for the fluorescamine labelling of proteins for electrophoresis was used in this study to label insulin and bovine serum albumin for rod gel electrophoresis:

1) $2.5 \times 10^{-2}$ g of protein was dissolved in 25 $cm^3$ of 0.025M phosphate buffer at pH 7.4.
2) 1 $cm^3$ of a 0.1% (w/v) solution of fluorescamine in acetone was added and the mixture shaken briefly. Fluorescence was seen to develop during the shaking procedure.

The labelled protein was stored at 4°C and used within one month of preparation.

**Appendix 2**

**Reproducibility of delivery by automatic replicating pipettes and bottle dispensing pipette**

Variable volume replicating pipettes (Gilson Pipetman, P100, P200, P1000 and P5000, Anachem) were used throughout this study for delivery of liquids in the range 20 $um^3$ to 5 $cm^3$. The reproducibility of delivery was assessed by weighing 10 consecutive deliveries of nominal volumes of distilled water; the following volumes were weighed using a 4 figure analytical balance (Mettler H15). 20, 40, 60, 80, 100, 150, 200, 400, 600, 800, 1000, 2000, 3000, 4000 and 5000 $um^3$. The coefficient variation did not exceed 0.69% in the range 100 to 5000 $um^3$ and was less than 1.87% in the range 20 to 100 $um^3$.

To assess the reproducibility of delivery of a nominal 4 $cm^3$ volume of scintillation fluid using a bottle dispensing pipette (Oxford dispenser IV), 5 consecutive deliveries of scintillant (section 2.2.3.) were weighed and the coefficient of variation for delivery was found to be 0.15%.

On the basis of the above results, it was considered that the reproducibility of delivery by automatic replicating and bottle pipettes was satisfactory.